

An Evolution-Inspired Strategy to Design Disulfide-Rich Peptides Tolerant to Extensive Sequence Manipulation

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1. Experimental Section

1.1 Materials and instruments

All the Fmoc-protected amino acids and Rink amide MBHA resin used for peptide synthesis were supplied by GL Biochem (Shanghai, China). Glutathione oxidized (GSSG), glutathione reduced (GSH) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were bought from Energy Chemical (Shanghai, China). $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, meta-phosphoric acid (HPO_3) and trypsin were purchased from Sigma-Aldrich (Beijing, China). Acetonitrile (ACN), trifluoroacetic acid (TFA), sodium acetate trihydrate ($\text{NaAc} \cdot 3\text{H}_2\text{O}$), EDTA disodium salt dihydrate, Sodium dodecyl sulfate (SDS), and all the chemicals used to prepare 1×TAE (Tris-HCl, EDTA disodium salt dihydrate, acetic acid), 1×TE (Tris-HCl, EDTA disodium salt dihydrate), neutralization buffer (Tris-HCl), elution buffer (glycine), 2YT medium (tryptone, yeast extract, NaCl), PEG/NaCl (PEG-6000, NaCl), binding buffer (Tris-HCl, NaCl, MgCl_2 , CaCl_2), washing buffer (Tris-HCl, NaCl, MgCl_2 , CaCl_2 , Tween-20) and blocking buffer (Tris-HCl, NaCl, MgCl_2 , CaCl_2 , Tween-20 and BSA) were purchased from Sigma-Aldrich (Beijing, China), Sangon Biotech (Shanghai, China) or Sinopharm Chemical Reagent (Beijing, China). Dimethyl sulfoxide (DMSO), acetic anhydride (Ac_2O), N,N-dimethylformamide (DMF), piperidine, and phenol were bought from Sinopharm Chemical Reagent (Beijing, China). Klenow fragment, *Sfi* I, *Not* I, T4 DNA Ligase, and 20 bp DNA Ladder were bought from Takara (Beijing, China). 20×PBS (Na_2HPO_4 , KH_2PO_4 , NaCl, KCl), DL5000 DNA Marker, ampicillin (Amp), agar, ammonium persulfate and 4S Red Plus were bought from Sangon Biotech (Shanghai, China). EZ-Link™ Sulfo-NHS-LC-Biotin, Dynabeads™ M-280 Tosylactivated, Dynabeads™ M-280 Streptavidin and NeutrAvidin protein were purchased from Thermo Fisher Scientific (Shanghai, China). Streptavidin was purchased from Biosynthesis Biotechnology (Beijing, China). Acetonitrile-D3 was purchased from Cambridge Isotope Laboratories (Beijing, China). Deuterium oxide (D_2O) was purchased from Energy Chemical (Shanghai, China). TG1 *E.coli* strain was purchased from Beyotime (Shanghai, China).

All peptides were synthesized using solid-phase peptide synthesis by a CEM Discover Liberty BLUE microwave-assisted peptide synthesizer. High performance liquid chromatography (HPLC, SHIMADZU) equipped with a quaternary solvent manager (QSM), an AQUITY[®] PDA detector and a column manager was used for analysis of peptides. Reaction progress was monitored by HPLC (a flow rate of 1.0 mL·min⁻¹ flow rate of H₂O (+0.1% TFA) and ACN (+0.1% TFA); isocratic with 10% ACN (+0.1% TFA) for 5.0 min followed by a linear gradient of 10% to 85% ACN (+0.1% TFA) over 30 min). A HITACHI U-3900H UV/Vis spectrometer was used for the quantification of peptides. Surface Plasmon Resonance (SPR) assays were performed by Biacore T200. Fluorescence polarization was recorded by Tecan Infinite[®] 200 PRO Microplate Reader. Bruker impact II-TOF mass spectrometry, Bruker Esquire 3000 plus ion trap ESI mass spectrometry and Bruker autoflex max MALDI-TOF mass spectrometry were applied to identify the formed peptides. NMR experiments were recorded at 298 K on Bruker AVANCE III 600 MHz and 850 MHz equipped with a cryogenic triple-resonance probe. Fast Protein Liquid Chromatography (FPLC) equipped with a Hitrap[™] Desalting column (GE Healthcare) was used to purify the biotinylated proteins.

1.2 Synthesis of peptides

All peptides are N-terminally acetylated and C-terminal amidated, which were synthesized at 0.05 mmol scale using the Fmoc solid-phase peptide synthesis (SPPS) on a CEM Liberty blue automated microwave peptide synthesizer (Table S1). Amino acids were coupled onto the MBHA resins using the standard coupling protocol. Peptides were cleaved from the resin and deprotected by treating with a TFA cleavage cocktail for 4 h at room temperature. Then, the cleaved peptides were precipitated in cold diethyl ether, and purified using a HPLC system. All peptides were purified to a purity of >95% before performing the oxidation (Figures S1 and S7).

1.3 Oxidation of peptides

Reduced peptides (>95% purity) isolated by HPLC were identified by mass spectrometry (Table S2). The HPLC-isolated peptides were lyophilized and re-dissolved in a mixture of water and acetonitrile (70%:30% vol/vol) as the stock solution for further use. Concentrations of the reduced

peptide stock solutions were determined by UV/Vis spectroscopy ($\epsilon_{\text{Trp}} = 5,502 \text{ cm}^{-1} \text{ M}^{-1}$ at 280 nm). In a typical experiment for peptide oxidation, the reduced peptide was reconstituted in an appropriate amount of phosphate buffer (100 mM, pH 7.4) containing 30% acetonitrile, 0.5 mM GSH and 0.5 mM GSSG to achieve a concentration of 50 μM . After about 4–6 h, the oxidation was complete, and the oxidized peptide was characterized by HPLC and mass spectrometry (Table S3). The Acn-protected peptides were oxidized in the redox buffer in the same way. After the HPLC-purification of the oxidized Acn-protected peptides, the peptide was lyophilized and reconstituted in pure water to achieve a definite concentration (determined by UV/Vis spectroscopy). Then, the peptide solution was diluted by addition of methanol with 0.1% TFA to a concentration of 50 μM , into which 10 eq. I dissolved in methanol was added dropwise. After ~ 1 h, the Acn was completely removed from the peptide, leading to the formation of the third disulfide bond in the peptide. The final Acn-deprotected product was analyzed using HPLC and mass spectrometry (Table S4). To characterize the disulfide pairing of oxidized **12** and **14**, the lyophilized powder (oxidized **12** and **14** isolated by HPLC) was dissolved in a phosphate buffer (100 mM, pH 6.0) containing 50 $\mu\text{g/mL}$ trypsin for 4 h, and then the major digested peptide fragment isolated by HPLC (monitored at 280 nm) was identified using mass spectrometry (Figures S9 and S10).

1.4 Construction of phage-displayed peptide libraries

The peptide libraries were constructed based on a procedure reported previously (Table S5).¹ In brief, DNA libraries encoding random peptide sequences were digested by *Sfi* I (10 h, 50 °C) and *Not* I (10 h, 37 °C), which were recovered by gel purification. Then, the purified DNA fragments were

ligated with gel-purified *Sfi* I/*Not* I-digested vector pCantab 5E (ratio of insert and vector: 10/1). The ligation mixture was then transformed into *E. coli* TG1 competent cells. The cells were plated on 2 × YT/ampicillin agar plates and incubated at 37 °C for 12 h. The size of the phage libraries was determined by measuring the total number of colonies. The colonies on the plates were then scraped off the plates and propagated for phage production and purification. 20–30 phage clones were randomly picked up for sequencing to evaluate the quality of the phage libraries.

1.5 Phage panning

Proteins (MDM2 and Bcl-2; 5 µM) were biotinylated in phosphate buffer saline (PBS; pH 7.4) with the addition of Sulfo-NHS-LC-biotin (50 µM) for ~0.5 h at room temperature. After the reaction, the unreacted Sulfo-NHS-LC-biotin was removed using a desalt column on an AKTA pure system (running buffer: pH 7.4 PBS). The biotinylated proteins can then be immobilized on streptavidin-coated and neutravidin-coated magnetic beads for screening as described previously.^{2,3} Briefly, 100 µL of streptavidin-coated magnetic beads was washed three times with binding buffer (150 mM NaCl, 1 mM CaCl₂, 10 mM Tris-HCl, 10 mM MgCl₂, pH 7.4) in a 1.5 mL microcentrifuge tube, which were then re-suspended with 100 µL binding buffer and distributed equally into two 1.5 mL microcentrifuge tubes. Then, the biotinylated protein (first round: 10 µg; second round: 5 µg; third round: 2 µg) was added into one of the two tubes; meanwhile, the same volume of 1 × PBS was added into the other one as a control. The two tubes were incubated on a slowly rotating vortex mixer for 15 min at room temperature. Then, the beads in the two tubes were washed three times with the binding buffer to remove the free proteins, which were then resuspended with 300 µL

binding buffer and 150 μ L Blocking buffer (binding buffer containing 0.3% Tween-20 and 3% w/v BSA). The suspension of beads was incubated at room temperature for 2 h on a slowly rotating vortex mixer. Meanwhile, to a tube containing 3.0 mL of phages ($>10^{12}$ t.u.) dissolved in binding buffer, 1.5 mL blocking buffer was added, and the solution was incubated at room temperature for 2 h on a slowly rotating vortex mixer. Then, the phages in solution were split equally into two tubes, into which the BSA-blocked beads with and without the immobilized proteins were added, respectively. After 30 min incubation at room temperature, the unbound phages in the supernatant were removed and the beads were washed for nine times with washing buffer (binding buffer containing 0.1% Tween-20) and twice with binding buffer. During the washing steps, the tubes were replaced at least three times with new ones to avoid the non-specific adsorption of phages. After the last washing step, the beads were resuspended with 200 μ L elution buffer (50 mM glycine, pH 2.2) and incubated at room temperature for 5 min. Then, the beads were magnetically precipitated and the supernatant was transferred to a new microcentrifuge tube containing 50 μ L neutralization buffer (1 M Tris-Cl, pH 8.9). The eluted phages from both the experimental and control group were diluted to infect exponentially growing TG1 cells to quantify the phage titer, and the eluted phages from the experimental group were propagated for the production of phages used for next-round panning. After three rounds of panning as described above, 20–30 phage clones were randomly picked for sequencing. Note that streptavidin-coated and neutravidin-coated magnetic beads were used to immobilize biotinylated proteins alternatively in each round of selection to prevent the enrichment of streptavidin and neutravidin binders during the panning.^{2,4} For the library screening against streptavidin, streptavidin-coated magnetic beads were directly used for panning.

1.6 Fluorescence polarization (FP) assays

FP assays were performed on 96-well flat-bottom OptiPlate black plates using a Tecan Infinite® 200 PRO Microplate Reader. To examine the binding affinity of oxidized **16** to Bcl-2, a fluorescein-labelled **16** (FITC- β Ala-**16**) was synthesized and purified to a purity of >95% using HPLC. Fluorescein-labelled **16** was first dissolved in DMSO and then diluted with PBS (pH 7.4) for further use. Sumo-Bcl-2 was obtained from an *E. coli* cell expression system described previously.⁵ For the FP assay, 20 nM of FITC- β Ala-**16** was incubated with Sumo-Bcl-2 (0–1.2 μ M) in PBS (pH 7.4) on a 96-well plate at room temperature for 10 min, and fluorescence anisotropies were recorded. The K_D value of the binding between FITC- β Ala-**16** and Bcl-2 can then be obtained by fitting the data with the single-site binding model. For the FP competition assay, 20 nM of FITC- β Ala-**16** and 400 nM of Sumo-Bcl-2 were incubated with oxidized **16** (0–10 μ M) under the same conditions as described above, and fluorescence anisotropies were recorded. The K_i value of the binding between oxidized **16** and Bcl-2 can then be obtained using a data-fitting procedure reported previously.⁵ The binding of peptides to MDM2 was evaluated using the same procedure described above, which has been described in our previous reports.^{1,6}

1.7 Surface plasmon resonance (SPR) assays

SPR assays were performed using a Biacore T200. Target proteins (Sumo-Bcl-2 and streptavidin) were immobilized on chip surface using a procedure described previously.⁵ Briefly, Flow cell Fc4 was activated first with a mixture of NHS and EDC, and then Sumo-Bcl-2 (or streptavidin) dissolved in 10 mM acetate buffer (pH 5.0) flowed through the cell for protein coupling

to reach a target response of 1000 RU. Flow cell Fc3 that was not immobilized with proteins was used as the reference channel. Serially diluted samples were then passed over the flow cells at a flow rate of 30 μ L/min (160, 320, 640, 1280, 2560, 640 nM for oxidized **15**, **16**, and **17**; 20, 40, 80, 160, 320, 80 nM for oxidized **11**, **15**, and **16**; 5, 10, 20, 40, 80 nM for oxidized **17**). Kinetics data were then analyzed using a 1:1 binding model and local fit to obtain kinetic rate constant and dissociation constant.

1.8 NMR characterization

NMR samples containing 0.5 mM of oxidized **15** and oxidized **16** were prepared in 50% perdeuterated acetonitrile and 50% H₂O. NMR experiments were recorded at 298 K on Bruker AVANCE III 600 MHz and 850 MHz equipped with a cryogenic triple-resonance probe for oxidized **15** and **16**, respectively. Two dimensional (2D) ¹H, ¹⁵N/¹³C HSQC spectra were recorded to obtain chemical shifts of ¹³C/¹⁵N in backbone and side-chains. 2D ¹H-¹H TOCSY (80 ms) and 2D ¹H-¹H COSY spectra were acquired to assign NMR signals of the peptide, and 2D ¹H-¹H NOESY spectra with a mixing time of 300 ms were measured to obtain ¹H-¹H distance constraints. The NMR data were processed using NMRPipe/NMRDraw and analyzed using NMRFAM-SPARKY.^{7,8} About 95% of all NOE cross-peaks were assigned manually. Prediction of backbone dihedral angle restraints of the oxidized **16** was performed using TALOS-N based on chemical shifts of backbone resonances. Structural calculations were carried out with ARIA2.3.2 and CNS1.21.⁹⁻¹¹ An ensemble of 10 lowest-energy structures was generated from a total of 150 structures. The structures were visualized in PyMol and the quality of structures was analyzed using PROCHECK.¹²

1.9 Proteolytic stability of peptides

100 μ M **15**-Ala, **16**-Ala, oxidized **15**, and oxidized **16** were respectively treated with phosphate buffer (pH = 7.4, 100 mM) containing chymotrypsin at 37 °C. The chymotrypsin concentration used for the digestion of **15**-Ala and oxidized **15** is 0.3 μ g/mL. The chymotrypsin concentration used for the digestion of **16**-Ala and oxidized **16** is 5.0 μ g/mL. At predefined times, 50 μ L aliquots were taken with a pipette, quenched with 70 μ L 10% HPO₃, and analyzed by HPLC.

1.10 CD characterization

Circular dichroism (CD) spectra were measured using a 1.0 mm path length cuvette at room temperature (25 °C). All measured peptides were dissolved in water to reach a concentration of 50 μ M. The baseline signal (pure aqueous solution) were subtracted from each spectrum. CD spectra were recorded in a wavelength range of 190–260 nm with a bandwidth of 2.0 nm, a data pitch of 1.0 nm, a response time of 8.0 s, and a scanning speed of 50 nm/min. The thermal denaturation CD spectra were recorded in a temperature range of 25–95 °C with a heating rate of 10 °C/min. For temperature melts, the change of ellipticity at 220 nm was monitored as temperature increased from 25 to 95 °C in an increment of 10 °C. Other experimental conditions were consistent with those of the general method.

2. Supplementary Data

2.1 Sequences and mass spectral characterizations of the synthesized peptides

Table S1. Sequences of the synthesized peptides

Name	Sequence
1	Ac-CGSGSGSGSGCPCWPELCPWIRSC-NH ₂
1-Acm	Ac-C(Acm)GSGSGSGSGCPC(Acm)WPELCPWIRSC-NH ₂
2	Ac-CGSGSGSGSGCPCICPELCPWIRSC-NH ₂
2-Acm	Ac-C(Acm)GSGSGSGSGCPCIC(Acm)PELCPWIRSC-NH ₂
3	Ac-CGSGSGSGSGCPCIWPCLPWIRSC-NH ₂
3-Acm	Ac-C(Acm)GSGSGSGSGCPCIWPC(Acm)LCPWIRSC-NH ₂
4	Ac-CGSGSGSGSGCPCIWPECCPWIRSC-NH ₂
4-Acm	Ac-C(Acm)GSGSGSGSGCPCIWPEC(Acm)CPWIRSC-NH ₂
5	Ac-CGSGSGSGSGCPCIWPELCPWIRSC-NH ₂
5-Acm	Ac-C(Acm)GSGSGSGSGCPCIWPELCP(Acm)IRSC-NH ₂
6	Ac-CGSGSGSGSGCPCIWPELCPWCRSC-NH ₂
6-Acm	Ac-C(Acm)GSGSGSGSGCPCIWPELCPW(Acm)RSC-NH ₂
7	Ac-CGSGSGSGSGCPCIWPELCPWICSC-NH ₂
7-Acm	Ac-C(Acm)GSGSGSGSGCPCIWPELCPWIC(Acm)SC-NH ₂
8	Ac-CGSGSGSGSGCPCIWPELCPWIRCC-NH ₂
8-Acm	Ac-C(Acm)GSGSGSGSGCPCIWPELCPWIRC(Acm)C-NH ₂
9	Ac-CGSGSGCPCIWPELCPWIRCC-NH ₂
9-Acm	Ac-C(Acm)GSGSGCPCIWPELCPWIRC(Acm) C-NH ₂
10	Ac-CGSGCPCIWPELCPWIRCC-NH ₂
10-Acm	Ac-C(Acm)GSGCPCIWPELCPWIRC(Acm) C-NH ₂
11	Ac-CGSGSGSGCGCPCIWPELCPWIRSC-NH ₂

11-Acm	Ac-C(Acm)GSGSGSGC(Acm)GCPCIWPELCPWIRSC-NH ₂
12	Ac-CGSGSGSGCGKGCPCIWPELCPWIRSC-NH ₂
13	Ac-CGSGSGSGSGCGPCIWPELCPWIRSCGGC-NH ₂
13-Acm	Ac-C(Acm)GSGSGSGSGCGPCIWPELCPWIRSCGGC(Acm)-NH ₂
14	Ac-GCPCIWPELCPWIRSCGKGCGSGSGSC-NH ₂
15	Ac-GCEKATESESPCPCIFPELCPWIVCC-NH ₂
15-Ala	Ac-GAEKATESESPAPAIFPELAPWIVAA-NH ₂
16	Ac-GCMILLDTDIWCPCSHPYACPENICC-NH ₂
16-Ala	Ac-GAMILLDTDIWAPASHPYAAPENIAA-NH ₂
FITC-βAla-16	FITC-(β-Ala)-GCMILLDTDIWCPCSHPYACPENICC-NH ₂
17	Ac-GCGSDEACFCPCSHPENCPMAQHC-NH ₂
17-Acm	Ac-G C(Acm)GSDEAC(Acm)FCPCSHPENCPMAQHC-NH ₂

Table S2. MS characterization of reduced peptides

Name	m/z expected	m/z found	Molecular formula
1	1287.5029 ([M+2H] ²⁺)	1287.4804	C ₁₀₅ H ₁₅₇ N ₃₁ O ₃₃ S ₆
1-Acm	1358.5400 ([M+2H] ²⁺)	1358.5229	C ₁₁₁ H ₁₆₇ N ₃₃ O ₃₅ S ₆
2	1251.0052 ([M+2H] ²⁺)	1250.9809	C ₁₀₀ H ₁₅₈ N ₃₀ O ₃₃ S ₆
2-Acm	1322.0423 ([M+2H] ²⁺)	1322.0223	C ₁₀₆ H ₁₆₈ N ₃₂ O ₃₅ S ₆
3	1279.5236 ([M+2H] ²⁺)	1279.4994	C ₁₀₆ H ₁₆₁ N ₃₁ O ₃₁ S ₆
3-Acm	1350.5607 ([M+2H] ²⁺)	1350.5439	C ₁₁₂ H ₁₇₁ N ₃₃ O ₃₃ S ₆
4	1287.5029 ([M+2H] ²⁺)	1287.4911	C ₁₀₅ H ₁₅₇ N ₃₁ O ₃₃ S ₆
4-Acm	1358.5400 ([M+2H] ²⁺)	1358.5289	C ₁₁₁ H ₁₆₇ N ₃₃ O ₃₅ S ₆
5	1251.0052 ([M+2H] ²⁺)	1250.9823	C ₁₀₀ H ₁₅₈ N ₃₀ O ₃₃ S ₆
5-Acm	1322.0423 ([M+2H] ²⁺)	1322.0474	C ₁₀₆ H ₁₆₈ N ₃₂ O ₃₅ S ₆
6	1287.5029 ([M+2H] ²⁺)	1287.4911	C ₁₀₅ H ₁₅₇ N ₃₁ O ₃₃ S ₆
6-Acm	1358.5400 ([M+2H] ²⁺)	1358.5389	C ₁₁₁ H ₁₆₇ N ₃₃ O ₃₅ S ₆
7	1265.9944 ([M+2H] ²⁺)	1265.9326	C ₁₀₅ H ₁₅₆ N ₂₈ O ₃₃ S ₆
7-Acm	1337.0315 ([M+2H] ²⁺)	1336.9771	C ₁₁₁ H ₁₆₆ N ₃₀ O ₃₅ S ₆
8	1300.5289 ([M+2H] ²⁺)	1300.6187	C ₁₀₈ H ₁₆₃ N ₃₁ O ₃₂ S ₆
8-Acm	1371.5660 ([M+2H] ²⁺)	1371.5506	C ₁₁₄ H ₁₇₃ N ₃₃ O ₃₄ S ₆
9	1156.4754 ([M+2H] ²⁺)	1156.4789	C ₉₈ H ₁₄₇ N ₂₇ O ₂₆ S ₆
9-Acm	1227.5125 ([M+2H] ²⁺)	1227.4617	C ₁₀₄ H ₁₅₇ N ₂₉ O ₂₈ S ₆
10	1084.4486 ([M+2H] ²⁺)	1084.4396	C ₉₃ H ₁₃₉ N ₂₅ O ₂₃ S ₆
10-Acm	1155.4858 ([M+2H] ²⁺)	1155.4882	C ₉₉ H ₁₄₉ N ₂₇ O ₂₅ S ₆
11	1300.5289 ([M+2H] ²⁺)	1300.6557	C ₁₀₈ H ₁₆₃ N ₃₁ O ₃₂ S ₆
11-Acm	1371.5660 ([M+2H] ²⁺)	1371.6973	C ₁₁₄ H ₁₇₃ N ₃₃ O ₃₄ S ₆
12	2728.1454 ([M+H] ⁺)	2728.169	C ₁₁₄ H ₁₇₅ N ₃₃ O ₃₃ S ₆
13	1401.0664 ([M+2H] ²⁺)	1401.2576	C ₁₁₅ H ₁₇₄ N ₃₄ O ₃₆ S ₆

13-Acm	1472.6039 ([M+2H] ²⁺)	1472.8245	C ₁₂₁ H ₁₈₄ N ₃₆ O ₃₈ S ₆
14	2785.1669 ([M+H] ⁺)	2785.292	C ₁₁₆ H ₁₇₈ N ₃₄ O ₃₄ S ₆
15	1444.1128 ([M+2H] ²⁺)	1444.0485	C ₁₂₄ H ₁₈₉ N ₂₉ O ₃₈ S ₆
15-Ala	2716.3670 ([M+Na] ⁺)	2716.5180	C ₁₂₄ H ₁₈₉ N ₂₉ O ₃₈
16	1472.1044 ([M+2H] ²⁺)	1472.0147	C ₁₂₅ H ₁₈₉ N ₃₁ O ₃₇ S ₇
16-Ala	2750.3683 ([M+H] ⁺)	2750.139	C ₁₂₅ H ₁₈₉ N ₃₁ O ₃₇ S
FITC-βAla-16	3363.2316 ([M+Na] ⁺)	3362.659	C ₁₄₇ H ₂₀₁ N ₃₃ O ₄₁ S ₈
17	2567.8820 ([M+H] ⁺)	2567.474	C ₁₀₀ H ₁₄₇ N ₃₁ O ₃₅ S ₇
17-Acm	2708.9536 ([M+H] ⁺)	2709.111	C ₁₀₆ H ₁₅₇ N ₃₃ O ₃₇ S ₇

Table S3. MS characterization of oxidized peptides

Name	m/z expected	m/z found	Molecular formula
1	1284.4794 ([M+2H] ²⁺)	1284.3791	C ₁₀₅ H ₁₅₁ N ₃₁ O ₃₃ S ₆
1-Acm	1356.5243 ([M+2H] ²⁺)	1356.4338	C ₁₁₁ H ₁₆₃ N ₃₃ O ₃₅ S ₆
2	1247.9817 ([M+2H] ²⁺)	1247.8865	C ₁₀₀ H ₁₅₂ N ₃₀ O ₃₃ S ₆
2-Acm	1320.0267 ([M+2H] ²⁺)	1319.9352	C ₁₀₆ H ₁₆₄ N ₃₂ O ₃₅ S ₆
3	1276.5001 ([M+2H] ²⁺)	1276.4056	C ₁₀₆ H ₁₅₅ N ₃₁ O ₃₁ S ₆
3-Acm	1348.5451 ([M+2H] ²⁺)	1348.4583	C ₁₁₂ H ₁₆₇ N ₃₃ O ₃₃ S ₆
4	1284.4794 ([M+2H] ²⁺)	1284.3811	C ₁₀₅ H ₁₅₁ N ₃₁ O ₃₃ S ₆
4-Acm	1356.5243 ([M+2H] ²⁺)	1356.4200	C ₁₁₁ H ₁₆₃ N ₃₃ O ₃₅ S ₆
5	1247.9817 ([M+2H] ²⁺)	1247.8832	C ₁₀₀ H ₁₅₂ N ₃₀ O ₃₃ S ₆
5-Acm	1320.0267 ([M+2H] ²⁺)	1320.0356	C ₁₀₆ H ₁₆₄ N ₃₂ O ₃₅ S ₆
6	1284.4794 ([M+2H] ²⁺)	1284.3877	C ₁₀₅ H ₁₅₁ N ₃₁ O ₃₃ S ₆
6-Acm	1356.5243 ([M+2H] ²⁺)	1356.5320	C ₁₁₁ H ₁₆₃ N ₃₃ O ₃₅ S ₆
7	1262.9709 ([M+2H] ²⁺)	1262.9076	C ₁₀₅ H ₁₅₀ N ₂₈ O ₃₃ S ₆
7-Acm	1335.0158 ([M+2H] ²⁺)	1334.9560	C ₁₁₁ H ₁₆₂ N ₃₀ O ₃₅ S ₆
8	1297.5054 ([M+2H] ²⁺)	1297.7093	C ₁₀₈ H ₁₅₇ N ₃₁ O ₃₂ S ₆
8-Acm	1369.5503 ([M+2H] ²⁺)	1369.5190	C ₁₁₄ H ₁₆₉ N ₃₃ O ₃₄ S ₆
9	1153.4519 ([M+2H] ²⁺)	1153.4468	C ₉₈ H ₁₄₁ N ₂₇ O ₂₆ S ₆
9-Acm	1225.4969 ([M+2H] ²⁺)	1225.4573	C ₁₀₄ H ₁₅₃ N ₂₉ O ₂₈ S ₆
10	1081.4252 ([M+2H] ²⁺)	1081.4202	C ₉₃ H ₁₃₃ N ₂₅ O ₂₃ S ₆
10-Acm	1153.4701 ([M+2H] ²⁺)	1153.4712	C ₉₉ H ₁₄₅ N ₂₇ O ₂₅ S ₆
11	1297.5054 ([M+2H] ²⁺)	1297.7083	C ₁₀₈ H ₁₅₇ N ₃₁ O ₃₂ S ₆
11-Acm	1370.0507 ([M+2H] ²⁺)	1370.2456	C ₁₁₄ H ₁₆₉ N ₃₃ O ₃₄ S ₆
12	2722.0985 ([M+H] ⁺)	2722.203	C ₁₁₄ H ₁₆₉ N ₃₃ O ₃₃ S ₆
13	1398.0429 ([M+2H] ²⁺)	1398.2554	C ₁₁₅ H ₁₆₈ N ₃₄ O ₃₆ S ₆

13-Acm	1470.5883 ([M+2H] ²⁺)	1470.8027	C ₁₂₁ H ₁₈₀ N ₃₆ O ₃₈ S ₆
14	2779.1200 ([M+H] ⁺)	2779.889	C ₁₁₆ H ₁₇₂ N ₃₄ O ₃₄ S ₆
15	1440.5889 ([M+2H] ²⁺)	1441.0	C ₁₂₄ H ₁₈₃ N ₂₉ O ₃₈ S ₆
16	1468.5805 ([M+2H] ²⁺)	1468.5018	C ₁₂₅ H ₁₈₃ N ₃₁ O ₃₇ S ₇
FITC-βAla-16	3357.1846 ([M+Na] ⁺)	3356.454	C ₁₄₇ H ₁₉₅ N ₃₃ O ₄₁ S ₈
17	2561.8351 ([M+H] ⁺)	2562.378	C ₁₀₀ H ₁₄₁ N ₃₁ O ₃₅ S ₇
17-Acm	2704.9223 ([M+H] ⁺)	2705.077	C ₁₀₆ H ₁₅₃ N ₃₃ O ₃₇ S ₇

Table S4. MS characterization of Acm-deprotected peptides

Name	m/z expected	m/z found	Molecular formula
1-Acm	1284.4794 ([M+2H] ²⁺)	1284.3796	C ₁₀₅ H ₁₅₁ N ₃₁ O ₃₃ S ₆
2-Acm	1247.9817 ([M+2H] ²⁺)	1247.8749	C ₁₀₀ H ₁₅₂ N ₃₀ O ₃₃ S ₆
3-Acm	1276.5001 ([M+2H] ²⁺)	1276.4077	C ₁₀₆ H ₁₅₅ N ₃₁ O ₃₁ S ₆
4-Acm	1284.4794 ([M+2H] ²⁺)	1284.3839	C ₁₀₅ H ₁₅₁ N ₃₁ O ₃₃ S ₆
5-Acm	1247.9817 ([M+2H] ²⁺)	1247.9731	C ₁₀₀ H ₁₅₂ N ₃₀ O ₃₃ S ₆
6-Acm	1284.4794 ([M+2H] ²⁺)	1284.4726	C ₁₀₅ H ₁₅₁ N ₃₁ O ₃₃ S ₆
7-Acm	1262.9709 ([M+2H] ²⁺)	1262.9082	C ₁₀₅ H ₁₅₀ N ₂₈ O ₃₃ S ₆
8-Acm	1297.5054 ([M+2H] ²⁺)	1297.5357	C ₁₀₈ H ₁₅₇ N ₃₁ O ₃₂ S ₆
9-Acm	1153.4519 ([M+2H] ²⁺)	1153.4463	C ₉₈ H ₁₄₁ N ₂₇ O ₂₆ S ₆
10-Acm	1081.4252 ([M+2H] ²⁺)	1081.3791	C ₉₃ H ₁₃₃ N ₂₅ O ₂₃ S ₆
11-Acm	1297.5054 ([M+2H] ²⁺)	1297.7097	C ₁₀₈ H ₁₅₇ N ₃₁ O ₃₂ S ₆
13-Acm	1398.0429 ([M+2H] ²⁺)	1398.2589	C ₁₁₅ H ₁₆₈ N ₃₄ O ₃₆ S ₆
17-Acm	2561.8351 ([M+H] ⁺)	2562.348	C ₁₀₀ H ₁₄₁ N ₃₁ O ₃₅ S ₇

2.2 Chromatograms of peptides

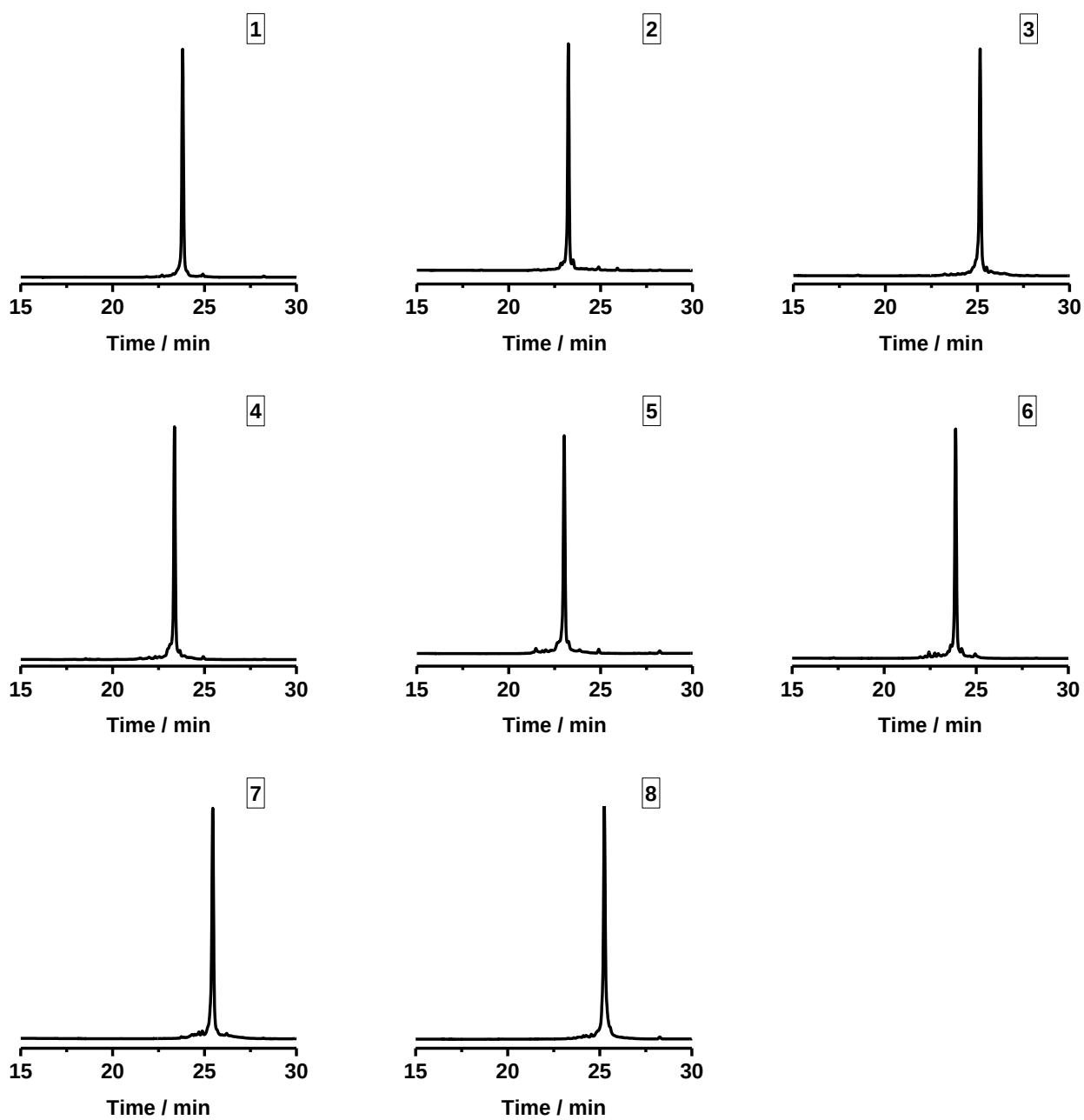


Figure S1. Chromatograms of peptides 1–8.

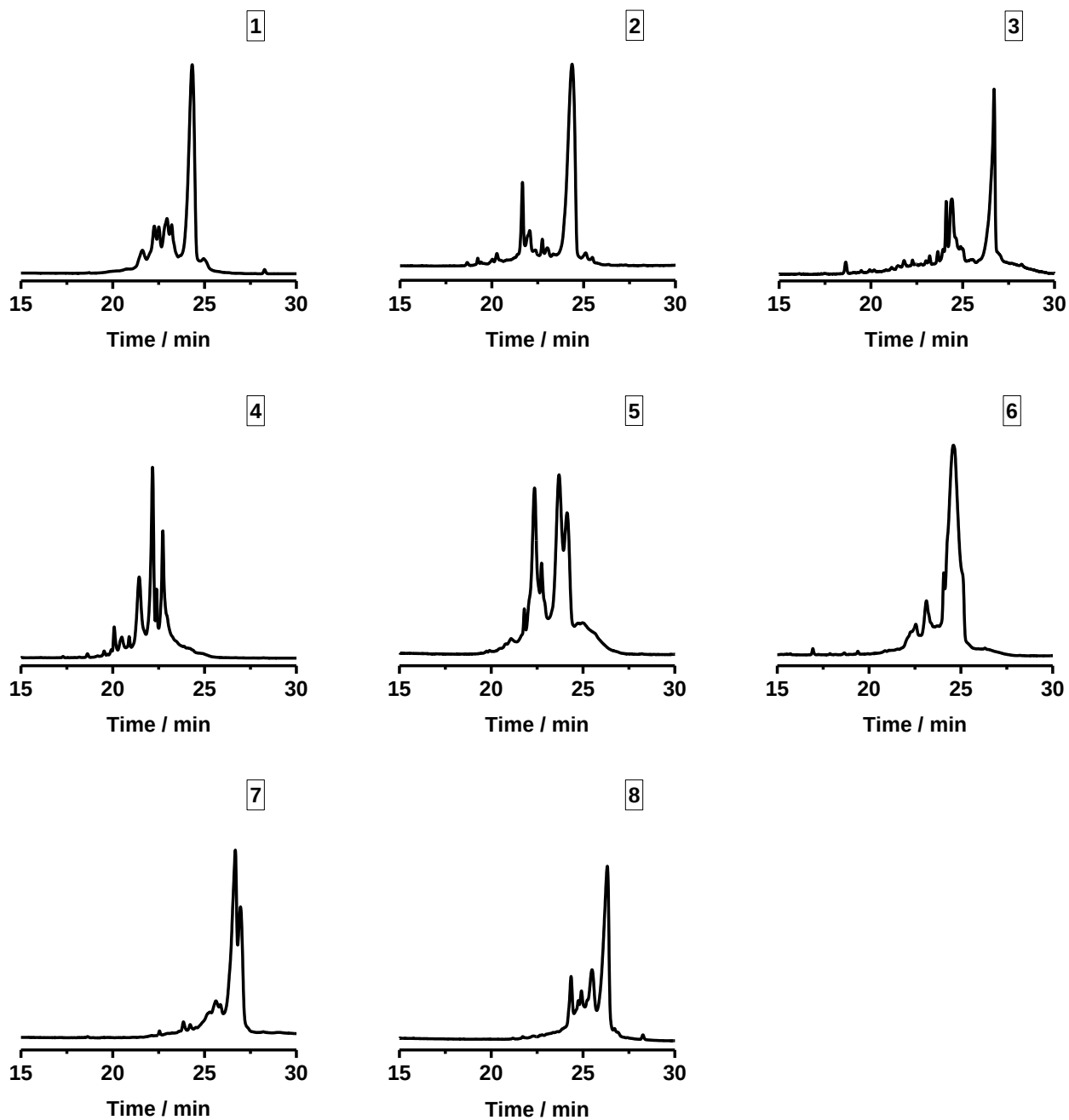


Figure S2. Chromatograms of the products formed after the oxidation of 1–8.

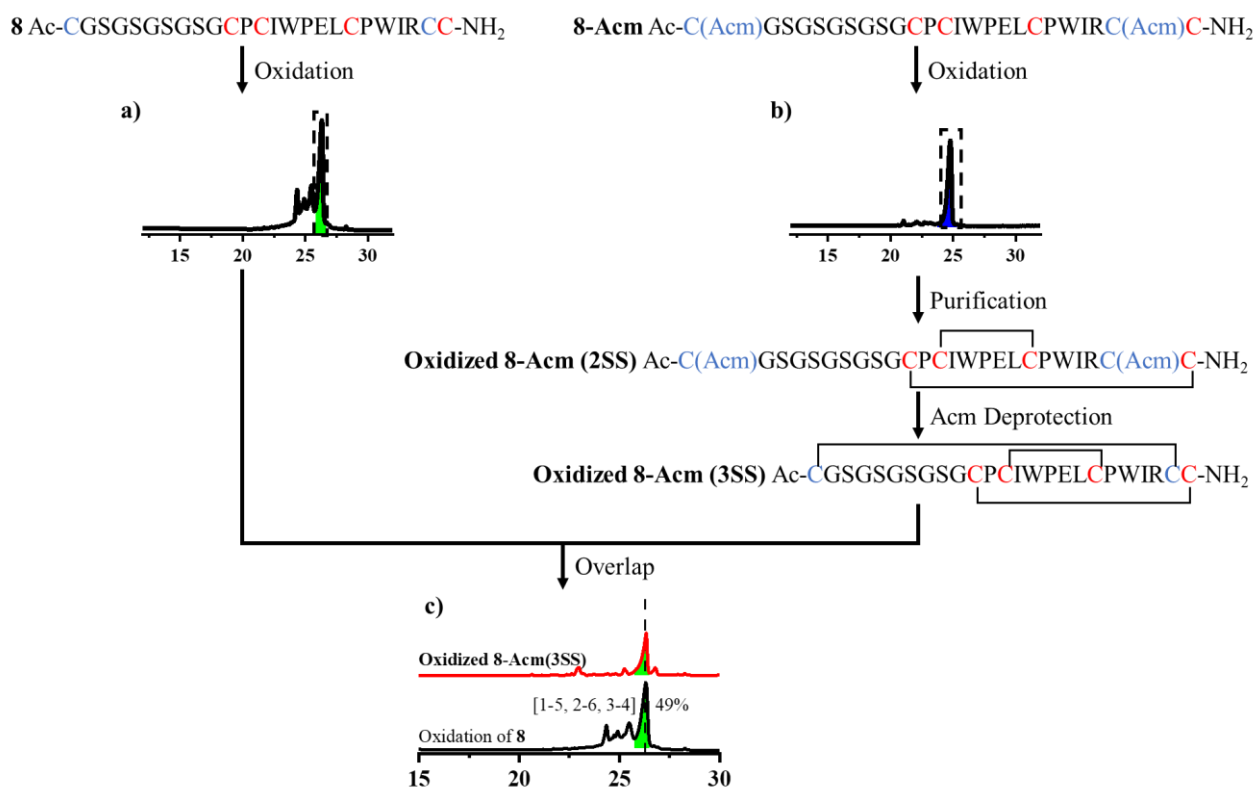


Figure S3. Analysis of disulfide pairing in peptide **8** through orthogonal protecting group strategies. a) Chromatogram of the products formed after the oxidation of **8**. b) Chromatograms of the products formed after the oxidation of **8-Acm**. c) Chromatogram of the products formed from the oxidative folding of **8** (black line) and chromatogram of the products formed from the Acm-deprotection of the oxidized **8-Acm** (red line).

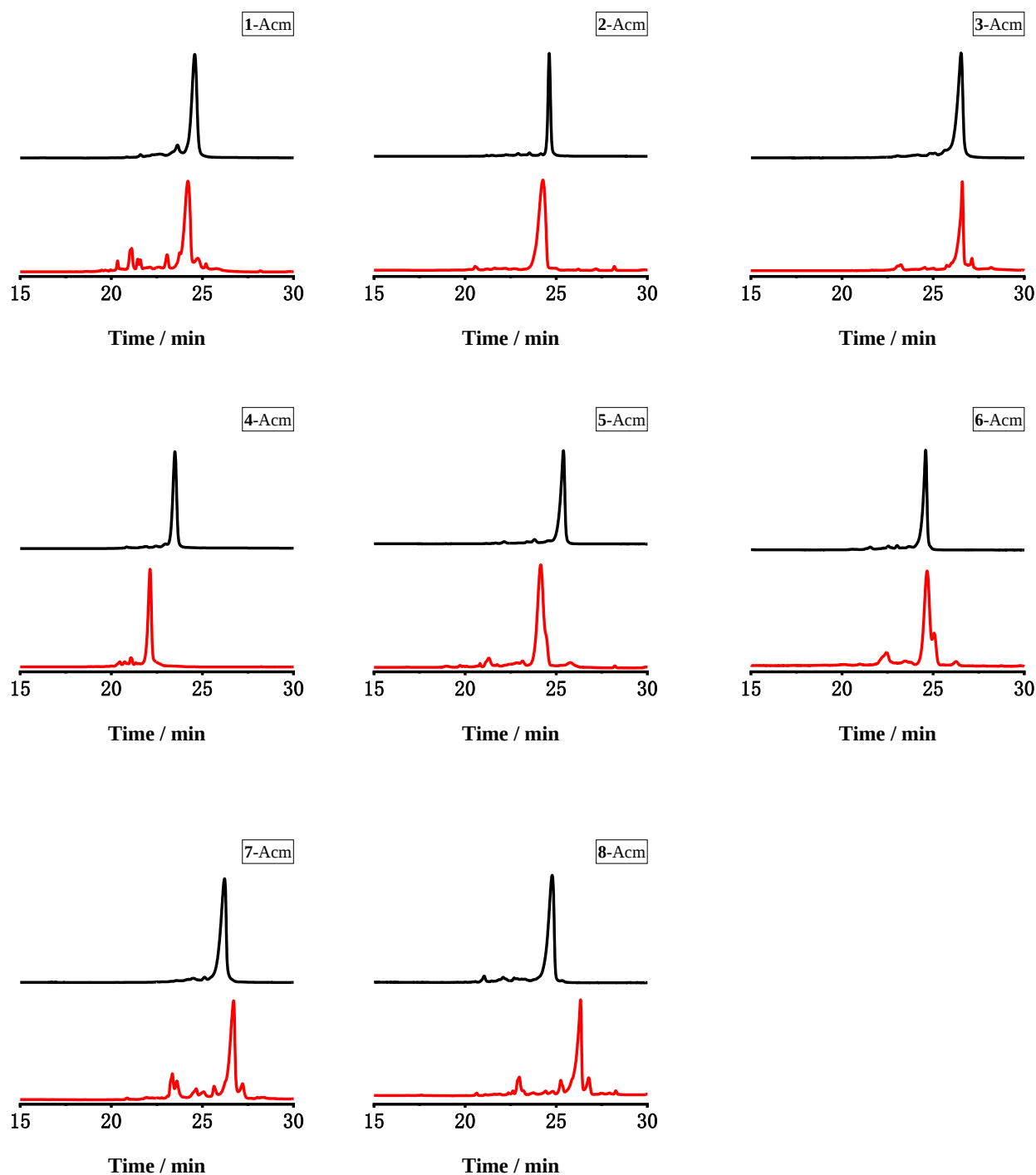


Figure S4. Chromatograms of products formed after the oxidation of **1-Acm~8-Acm** (black line) and chromatograms of the products formed after the Acm-deprotection of the oxidized **1-Acm~8-Acm** (red line).

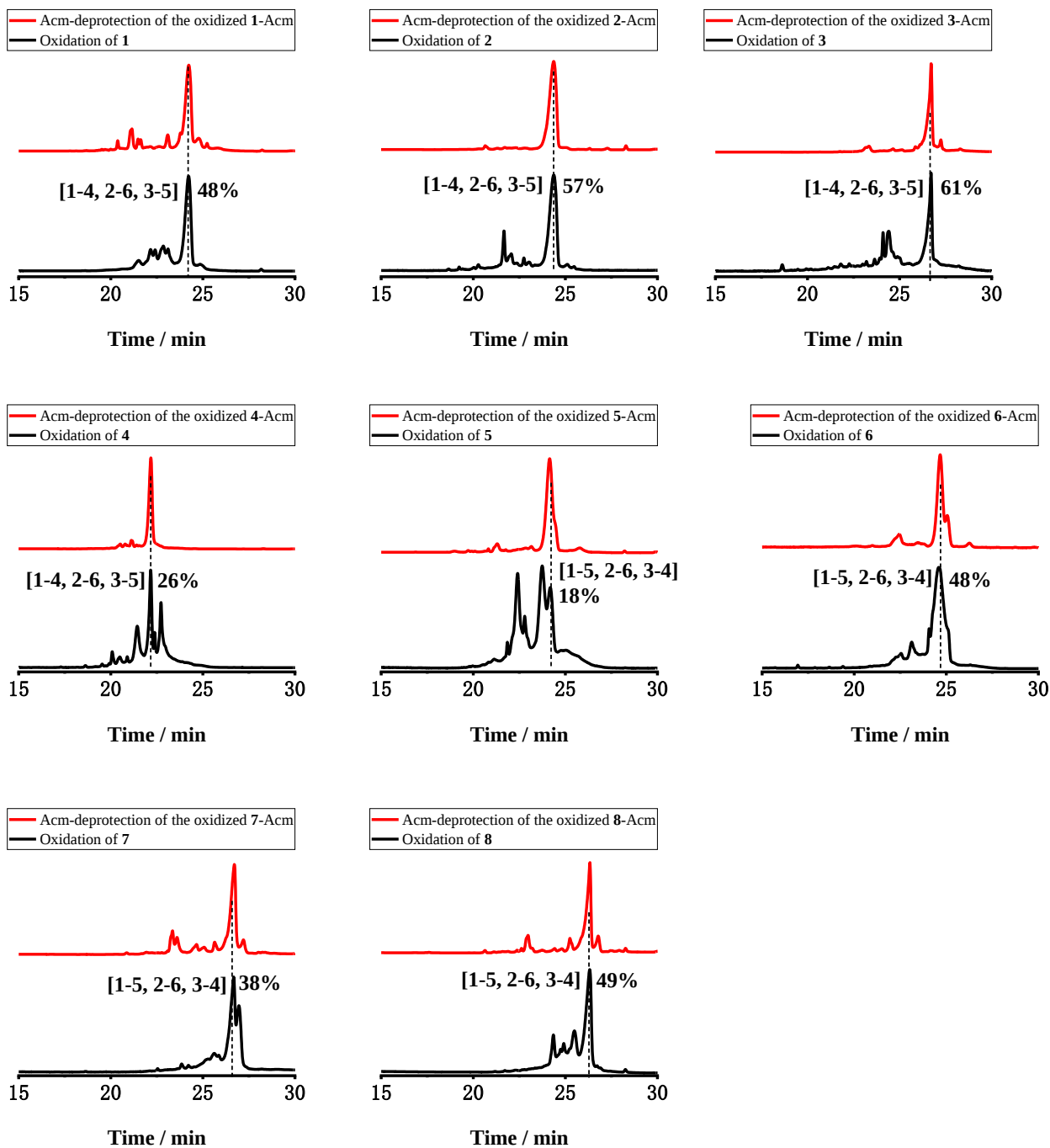


Figure S5. Chromatograms of products formed after the oxidation of **1**–**8** (black line) and chromatograms of the products formed after the Acm-deprotection of the oxidized **1-Acm~8-Acm** (red line).

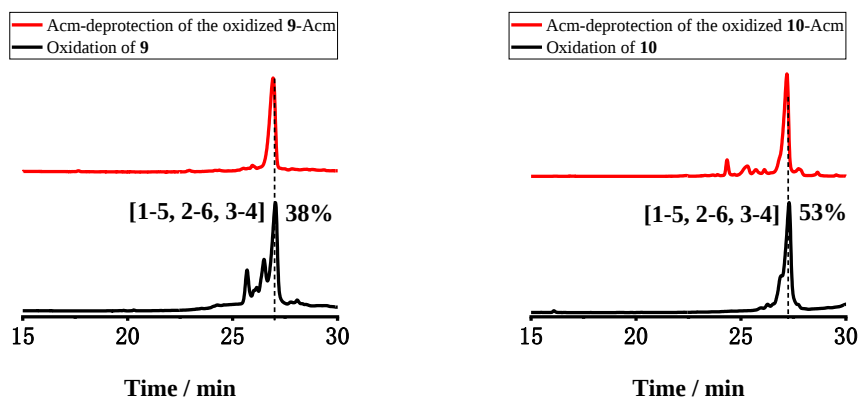


Figure S6. Chromatograms of the products formed after the oxidation of **9** and **10** (black line) and chromatograms of the products formed after the Acm-deprotection of the oxidized **9-Acm** and oxidized **10-Acm** (red line).

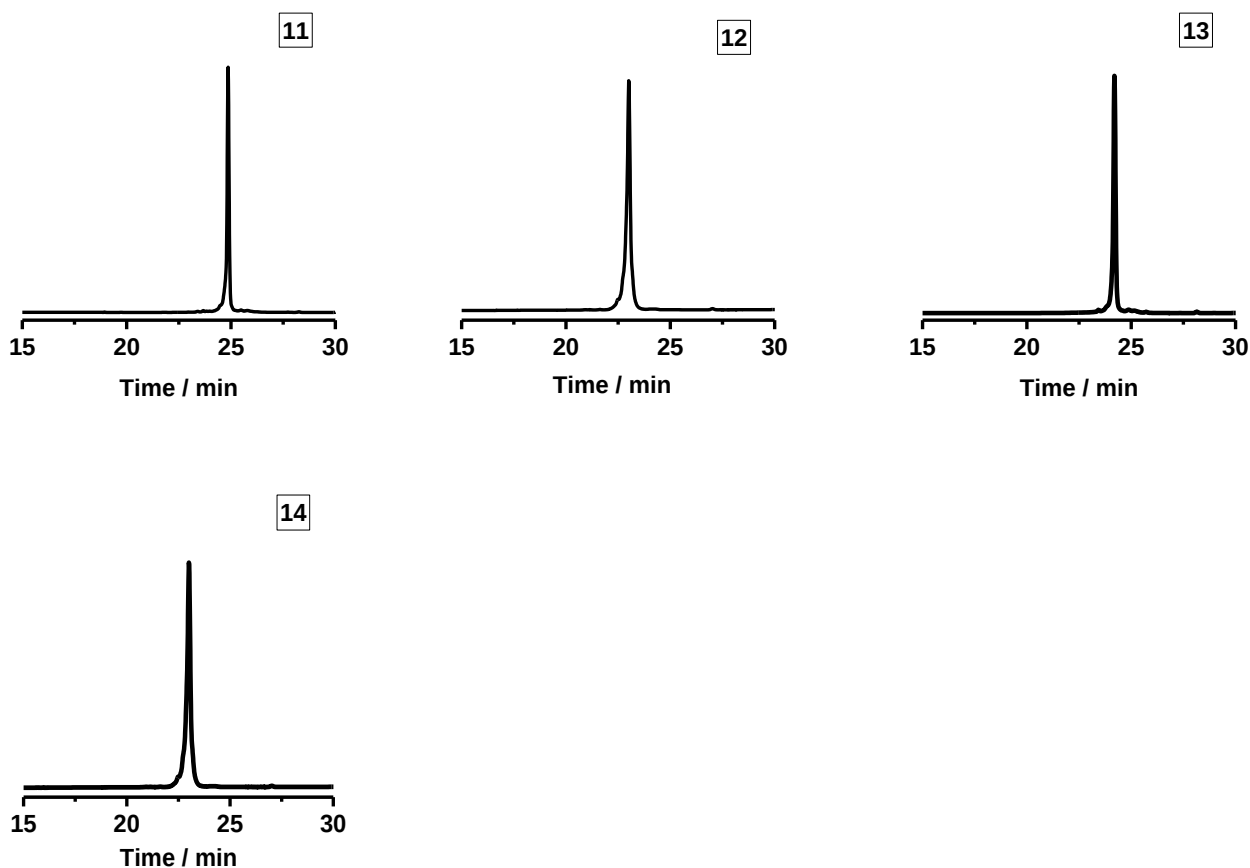


Figure S7. Chromatograms of peptides **11–14**.

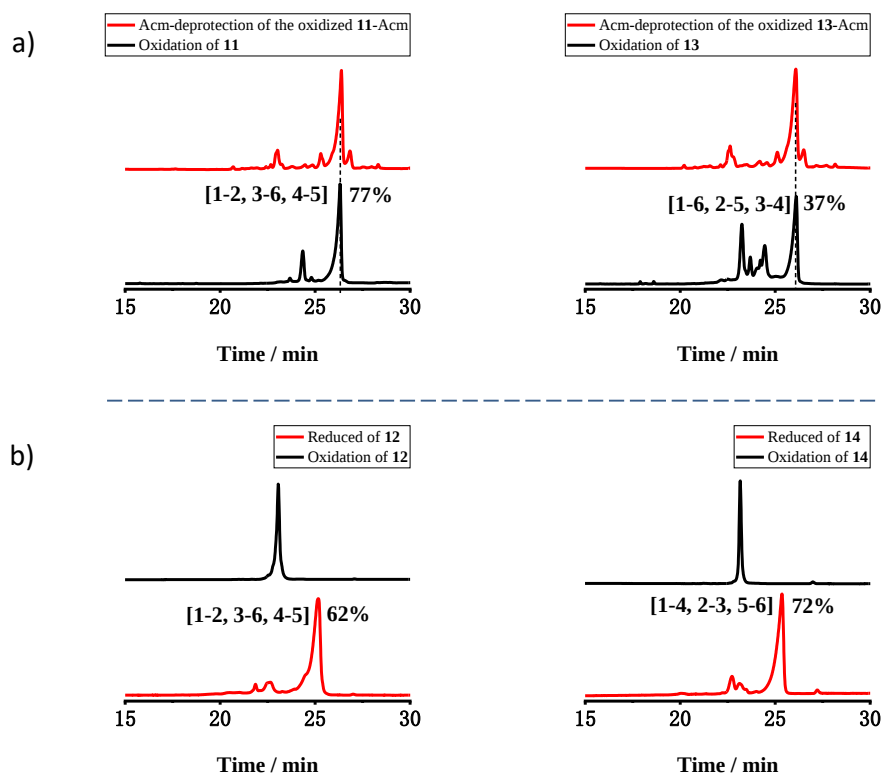


Figure S8. a) Chromatograms of the products formed after the oxidation of **11** and **13** (black line) and chromatograms of the products formed after the Acm-deprotection of oxidized **11-Acm** and oxidized **13-Acm** (red line). b) Chromatograms of **12** and **14** (black line) and chromatograms of the products formed after the oxidation of **12** and **14** (red line).

2.3 Characterization of disulfide pairing of 12 and 14 using trypsin digestion

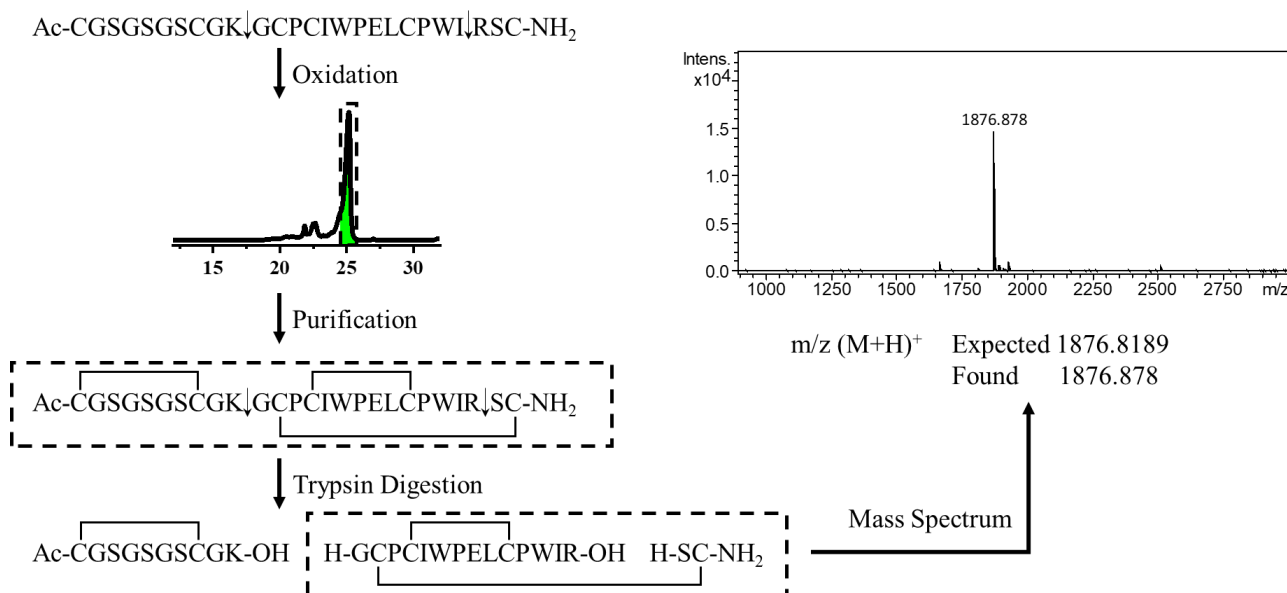


Figure S9. Tryptic digestion analysis of the oxidized **12**. ↓ denotes the cleavage site of trypsin.

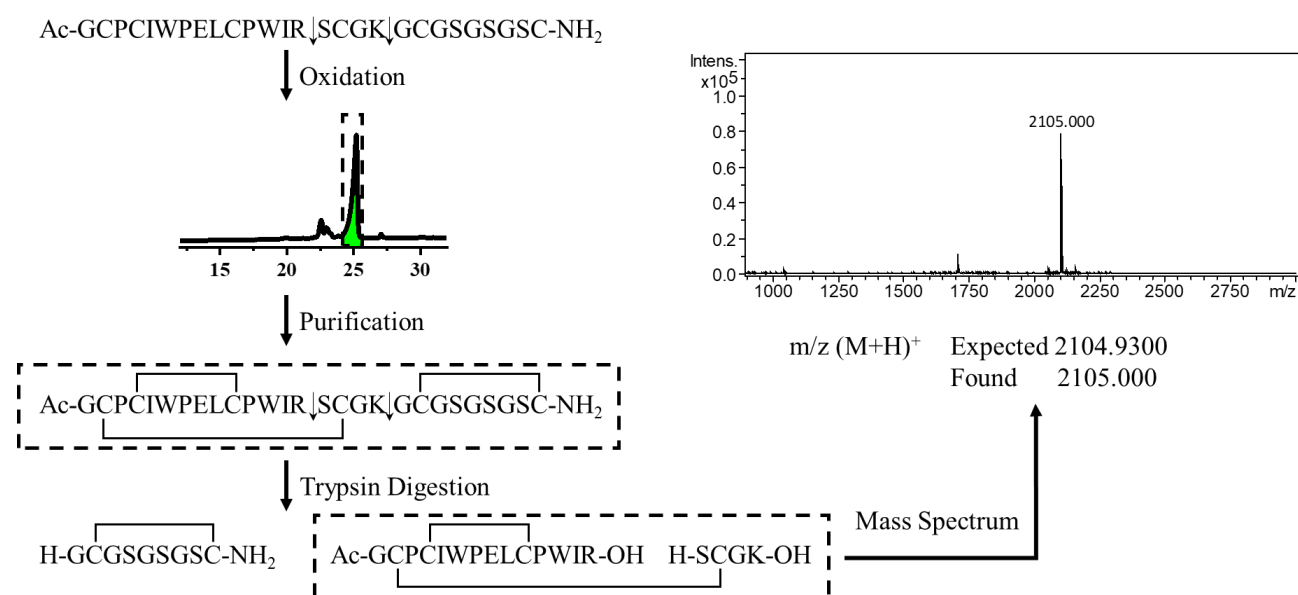


Figure S10. Tryptic digestion analysis of the oxidized **14**. ↓ denotes the cleavage site of trypsin.

2.4 Construction of phage-displayed peptide libraries

We selected **8** and **11** as model scaffolds to construct phage-displayed peptide libraries.

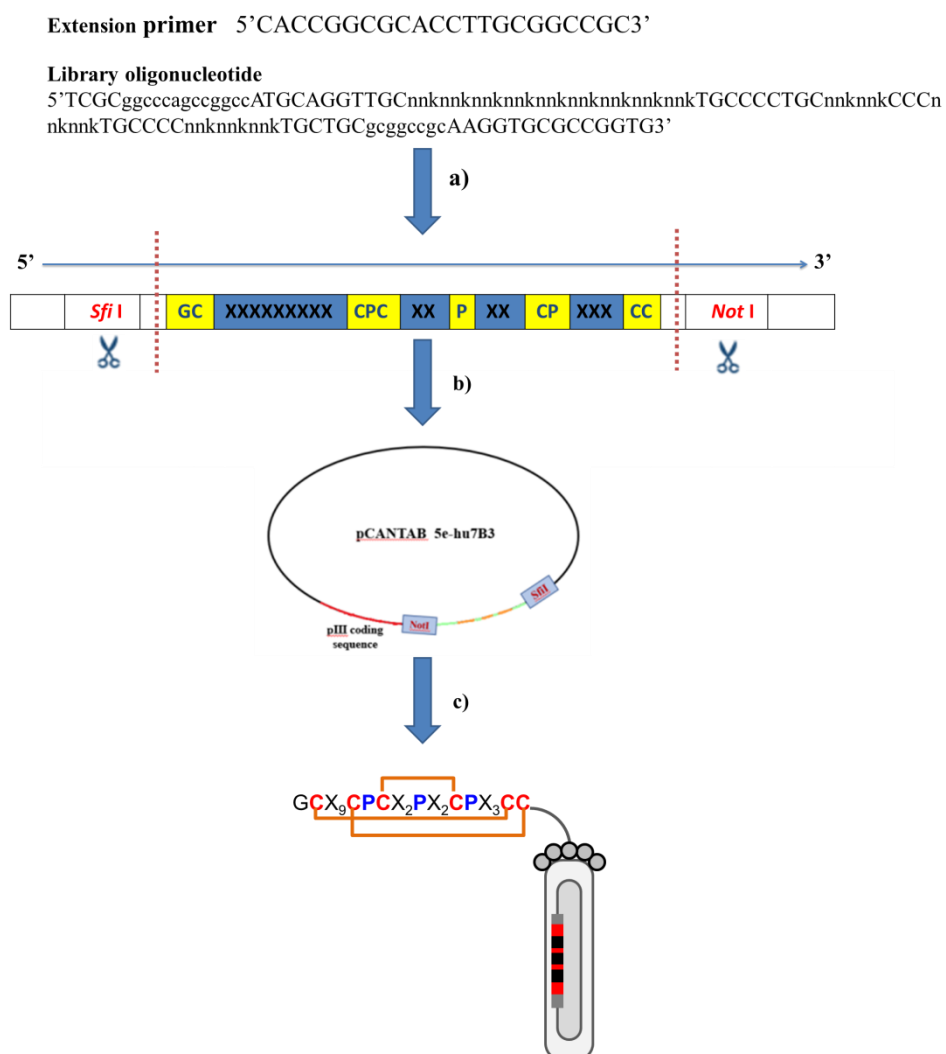


Figure S11. Design of phage-displayed disulfide-rich peptide library (taking **8-X₁₆** as an example).

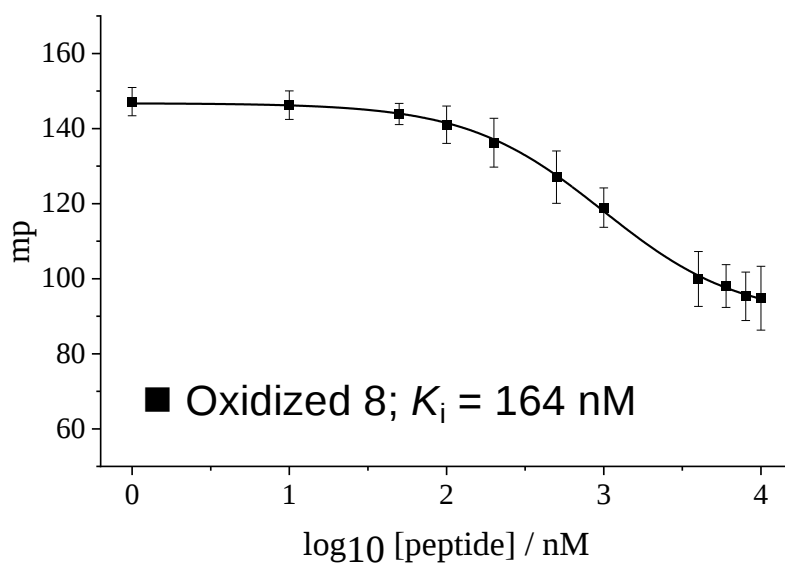


Figure S12. A fluorescence polarization competition assay showing the binding of oxidized **8** to MDM2.

Table S5. Four phage-displayed DRP libraries

Phage library	The sequence of phage library	Phage library capacity
8-X₅	GCX ₅ CPCIWPELCPWIRCC	4.75×10^8
8-X₉	GCX ₉ CPCIWPELCPWIRCC	1.4×10^9
8-X₁₆	GCX ₉ CPCX ₂ P X ₂ CPX ₃ CC	1.10×10^9
11-X₁₄	GCX ₅ CXCPCX ₂ PX ₂ CPX ₄ C	6.25×10^8

2.5 Sequences obtained from screening of peptide libraries and NMR characterization of the oxidized peptides

a) Clones	Sequence	b) Clone	Sequence
M-1	GC ERTDS CPCIWPELCPWIRCC	M-1	GC NLKLPEDSE CPCIWPELCPWIRCC
M-2	GC SDGES CPCIWPELCPWIRCC	M-2	GC SSYIMLCIH CPCIWPELCPWIRCC
M-3	GC EHGDS CPCIWPELCPWIRCC	M-3	GC RYDREHLSE CPCIWPELCPWIRCC
M-4	GC NADES CPCIWPELCPWIRCC	M-4	GC DNNLDSSLE CPCIWPELCPWIRCC
M-5	GC NDECS CPCIWPELCPWIRCC	M-5	GC SESVAFSYH CPCIWPELCPWIRCC
M-6	GC NESES CPCIWPELCPWIRCC	M-6	GC DDLNVFECI CPCIWPELCPWIRCC
M-7	GC NTEEP CPCIWPELCPWIRCC	M-7	GC ELSSNRVCD CPCIWPELCPWIRCC
M-8	GC NADNL CPCIWPELCPWIRCC	M-8	GC HMEGALTCE CPCIWPELCPWIRCC
M-9	GC NDIDE CPCIWPELCPWIRCC	M-9	GC GNSEDDSQR CPCIWPELCPWIRCC
M-10	GC QSEEL CPCIWPELCPWIRCC	M-10	GC SEFNHSSRS CPCIWPELCPWIRCC
M-11	GC QESDG CPCIWPELCPWIRCC	M-11	GC RKSPENECA CPCIWPELCPWIRCC
M-12	GC QEDEV CPCIWPELCPWIRCC	M-12	GC PSDIENEES CPCIWPELCPWIRCC
M-13	GC QERPA CPCIWPELCPWIRCC	M-13	GC EHVTFLLDCD CPCIWPELCPWIRCC
M-14	GC QAPGE CPCIWPELCPWIRCC	M-14	GC NQETSEINQ CPCIWPELCPWIRCC
M-15	GC TGSDE CPCIWPELCPWIRCC	M-15	GC VEEVTRES D CPCIWPELCPWIRCC
M-16	GC TYGSE CPCIWPELCPWIRCC	M-16	GC ELNTDNHCN CPCIWPELCPWIRCC
M-17	GC PSKVE CPCIWPELCPWIRCC	M-17	GC EASPDREID CPCIWPELCPWIRCC
M-18	GC LRAEE CPCIWPELCPWIRCC	M-18	GC KYATIRECE CPCIWPELCPWIRCC
M-19	GC WDERE CPCIWPELCPWIRCC	M-19	GC NEPHNANCA CPCIWPELCPWIRCC
M-20	GC DGSKE CPCIWPELCPWIRCC	M-20	GC APTSLSSCE CPCIWPELCPWIRCC
M-21	GC CQQAE CPCIWPELCPWIRCC	M-21	GC GQSTHNSCE CPCIWPELCPWIRCC
M-22	GC GAYTE CPCIWPELCPWIRCC	M-22	GC LEDVNYRTN CPCIWPELCPWIRCC
M-23	GC GEETE CPCIWPELCPWIRCC	M-23	GC DLEIMICQN CPCIWPELCPWIRCC
M-24	GC SEEDP CPCIWPELCPWIRCC	M-24	GC SVTNHEEED CPCIWPELCPWIRCC
M-25	GC MEPSE CPCIWPELCPWIRCC		
M-26	GC ETPDE CPCIWPELCPWIRCC		
M-27	GC YKDEA CPCIWPELCPWIRCC		

Figure S13. a) Sequences obtained from screening of **8-X₅** against MDM2 after three rounds of panning.

b) Sequences obtained from screening of **8-X₉** against MDM2 after three rounds of panning.

Clones			Sequences						
M-1	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-2	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-3	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-4	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-5	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-6	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-7	GC	EKATESESP	CPC	IF	P	EL	CP	WIV	CC
M-8	GC	AEGVEKIIK	CPC	IS	P	VM	CP	WLP	CC
M-9	GC	AEGVEKIIK	CPC	IS	P	VM	CP	WLP	CC
M-10	GC	AEGVEKIIK	CPC	IS	P	VM	CP	WLP	CC
M-11	GC	EVEVRAVCL	CPC	FY	P	SF	CP	WIK	CC
M-12	GC	EVEVRAVCL	CPC	FY	P	SF	CP	WIK	CC
M-13	GC	SAVEHVHAS	CPC	IL	P	EL	CP	WLT	CC
M-14	GC	REDSDERNG	CPC	NP	P	FL	CP	WIN	CC
M-15	GC	RSDEQMMDE	CPC	PV	P	YL	CP	WLP	CC
M-16	GC	STLQSRKME	CPC	PV	P	IM	CP	WLR	CC
M-17	GC	ASCADAYIE	CPC	VD	P	HF	CP	WMS	CC
M-18	GC	TIREVDREG	CPC	PL	P	QL	CP	WIQ	CC
M-19	GC	ISPENRHAE	CPC	ID	P	LL	CP	WLV	CC
M-20	GC	REQAVWVVG	CPC	LF	P	EL	CP	WIV	CC
M-21	GC	AEGVENIIK	CPC	IS	P	VM	CP	WLP	CC
M-22	GC	TKCNKVTG	CPC	YY	P	IM	CP	WLS	CC
M-23	GC	HQEGTIVES	CPC	EE	P	LL	CP	WIF	CC
M-24	GC	CGEASNSSE	CPC	SP	P	SL	CP	WLP	CC

Figure S14. Sequences obtained from screening of **8-X₁₆** against MDM2 after three rounds of panning.

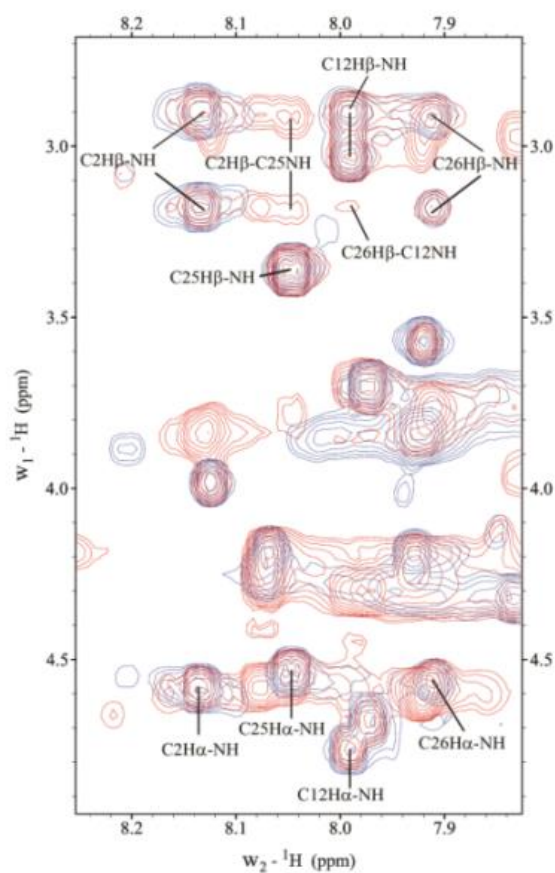
Table S6. Chemical shifts of oxidized **15** (BMRB 50900)

Residue	N	H _N	C α	H α	C β	H β	Other
G1	112.7	7.927	42.85	3.838			
C2		8.133		4.584	38.33	3.178, 2.292	
E3	120.6	8.082			25.96	2.029, 1.906	30.1 (C γ), 2.375 (H γ 2)
K4	120.6	7.929		4.21	22.04	1.325	26.39 (C δ), 1.589 (H δ 2), 24.62 (C γ), 2.892 (H ϵ 2), 1.548 (H γ 2), 0.914 (H γ 3), 7.344 (H ζ 1)
A5	123.7	8.074		4.255	16.69	1.352	
T6	109.6			4.279	57.28	3.555	18.96 (C γ 2), 1.11 (H ζ 21), 7.641 (H ν)
E7	119.2	8.07		4.183	25.59	2.085, 1.95	2.389 (H γ 2)
S8	112.6	7.787	55.76	4.316	61.3	3.79, 3.724	
E9	119.8	7.708	52.94	4.299	26.43	2.027, 1.88	2.357 (H γ 2)
S10	115.8	7.971	52.85	4.675	61.34	3.703	
P11				4.306	29.29	2.168, 1.867	47.85 (C δ), 3.689 (H δ 2), 3.621 (H δ 3)
C12		7.99	49.79	4.763	39.42	3.027, 2.901	
P13				4.675			48.15 (C δ), 24.13 (C γ), 3.877 (H δ 2), 3.71 (H δ 3), 2.012 (H γ 2), 1.805 (H γ 3)
C14	121	7.08	52.59	4.598	41.14	2.979	
I15	122.8	7.921	60.92	3.57	35.24	1.431	10.47 (C δ 1), 25.17 (C γ 1), 14.27 (C γ 2), 0.7076 (H δ 11), 1.191 (H γ 11), 0.9895 (H γ 12), 0.3744 (H γ 21)
F16		7.101	51.09	5.082	36.02	2.963	7.525 (H δ 2), 7.452 (H ϵ 2), 7.123 (H ζ)
P17			63.34	3.914	30.06	2.51, 1.931	48.2 (C δ), 3.815 (H δ 2), 3.792 (H δ 3)
E18	113.2	8.126	54.98	3.972	24.71	1.998, 1.934	30.47 (C γ), 2.426 (H γ 2)
L19	115.6	7.83	51.67	4.32	38.67	2.002, 1.767	22.85 (C δ 1), 19.62 (C δ 2), 24.64 (C γ), 0.9517 (H δ 11), 0.8025 (H δ 21), 1.597 (H γ)
C20	115.4	7.445	49.32	4.447	40.97	3.01	
P21			61.76	4.119	28.85	2.015, 1.519	47.58 (C δ), 24.3 (C γ), 3.265 (H δ 2), 1.763 (H γ 2), 1.407 (H γ 3)
W22	115	6.761	54.31	4.591	25.59	3.325, 3.173	7.054 (H δ 1), 10.04 (H ϵ 1), 7.255 (H ϵ 3), 7.191 (H η 2), 7.191 (H ζ 2), 7.243 (H ζ 3), 129.5 (N ϵ 1)
I23	127	8.281	62.74	3.766	18.85	1.107	10.38 (C δ 1), 30.42 (C γ 1), 18.26 (C γ 2), 0.8415 (H δ 11), 1.757 (H γ 12), 1.615 (H γ 13), 0.944 (H γ 21)
V24	117.2	7.076		4.19	35.24	1.737	15.33 (C γ 1), 17.16 (C γ 2), 0.9086 (H δ 11), 1.116 (H γ 21)
C25		8.047	52.8	4.537	40.23	3.364	
C26		7.912	53.38	4.566	36.22	3.179, 2.919	

Table S7. Chemical shifts of oxidized **16** (BMRB 50871)

Residue	N	H _N	C α	H α	C β	H β	Other
G1		7.833	42.85	3.791			
C2	116.7	8.007	52.87	4.478	38.03	3.051, 2.884	
M3	120.3	8.142	53.77	4.324	30.39	1.993, 1.956	29.59 (C γ), 2.526 (H γ 2), 2.435 (H γ 3)
I4	118.2	7.549	58.67	4.082	36.2	1.792	10.46 (C δ), 0.793 (H δ), 24.9 (C γ 1), 1.393 (H γ 12), 1.094 (H γ 13), 14.84 (C γ 2), 0.807 (H γ 22)
L5	122.6	7.724	52.23	4.335	39.74	1.568, 1.484	22.43 (C δ 1) , 0.8367 (H δ 12), 20.86 (C δ 2), 0.7793 (H δ 22), 24.42 (C γ)
L6	120.1	7.853		4.205	39.61	1.632, 1.516	22.49 (C δ 1), 20.77 (C δ 2), 0.8307 (H δ 12), 0.793 (H δ 22)
D7		8.005	50.83	4.581	35.15	2.894, 2.798	
T8	111.3	7.665		4.226	66.96	4.18	18.91 (C γ 2) , 1.113 (H γ 22)
D9	118.9	8.002	50.66	4.623	35.48	2.811, 2.763	
I10	117.6	7.516	59.05	3.989	36.14	1.624	10.57 (C δ 1), 0.6572 (H δ 12), 24.28 (C γ 1), 1.101 (H γ 12), 0.9157 (H γ 13), 14.63 (C γ 2), 0.5924 (H γ 22)
W11	121.1	7.655	54.06	4.62	27.27	3.253, 3.018	7.121 (H δ 1), 9.891 (H ϵ 1), 7.535 (H ϵ 3), 7.097 (H η 2), 7.356 (H ζ 2), 7.02 (H ζ 3),
C12	112.3	7.843	50.23	4.79	39.22	3.02, 2.952	
P13			61.04	4.26	29.06	1.996, 1.91	24.2 (C γ), 1.793 (H γ 3), 3.581 (H δ 2), 3.356 (H δ 3)
C14		7.374	52.04	4.662	37.68	3.187, 2.877	
S15	117.7	8.208	57.16	4.094	60.64	3.688, 3.664	
H16	116.8	7.335	49.68	4.913	26.37	2.842	7.271 (H δ 2), 8.498 (H ϵ 1)
P17			62.2	4.118	29.21	2.125, 1.758	48.1 (C δ), 3.714 (H δ 2), 3.606 (H δ 3), 24.79 (C γ), 1.956 (H γ 2), 1.911 (H γ 3)
Y18	118	8.07		4.122	33.85	3.102, 3.078	7.003 (H δ 2)
A19	121.3	7.696	49.95	4.28	17	1.313	
C20	112.3	7.857	50.25	4.934	39.84	3.213, 3.04	
P21			60.88	4.351	28.89	2.186	47.7 (C δ), 3.559 (H δ 2), 3.52 (H δ 3), 24.75 (C γ), 1.926 (H γ 2)
E22	117	7.965		4.218	25.83	2.071, 1.962	29.94 (C γ), 2.358 (H γ 2)
N23		7.925	50.7	4.598	36.42	2.769, 2.718	7.333 (H δ 21), 6.543 (H δ 22), 110.9 (N δ 2)
I24	121.7	7.928	59.62	4.151	36	1.841	10.87 (C δ), 0.8257 (H δ), 24.93 (C γ 1), 1.428 (H γ 12), 1.164 (H γ 13), 15.14 (C γ 2), 0.8938 (H γ 22)
C25	118.4	8.425	52.32	4.611	39.8	3.204, 2.857	
C26	118	8.039	53.06	4.465	39.72	3.216, 3.023	

a)



b)

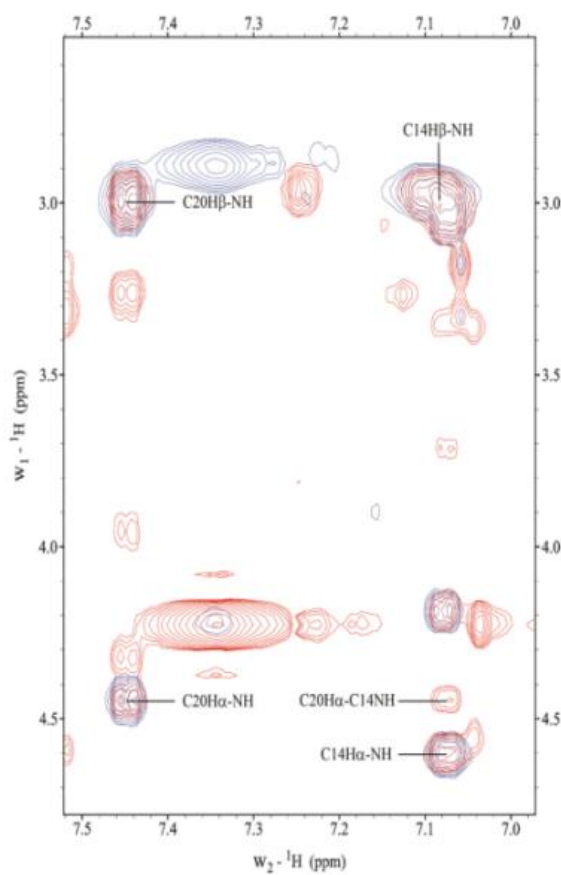


Figure S15. NMR characterization of oxidized **15**. a) and b) 2D ^1H - ^1H NOESY (red) and TOCSY (blue) spectra of oxidized **15** in ACN/ H_2O (50%/50%, v/v). Only local regions were plotted to show cross peaks of cysteine residues (disulfide connectivity: Cys 2-Cys 25, Cys 12-Cys 16, Cys 14-Cys 20) and cysteine-related assignments were labeled (data deposited in BMRB, accession number 50900).

Clones			Sequences						
B-1	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-2	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-3	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-4	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-5	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-6	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-7	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-8	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-9	GC	MILLDTDIW	CPC	SH	P	YA	CP	ENI	CC
B-10	GC	MILLDTDIW	CPC	SH	P	YA	CP	YNI	CC
B-11	GC	WTAMTLSAR	CPC	ER	P	HG	CP	RGC	CC
B-12	GC	FRKSPRLEV	CPC	AE	P	RA	CP	RGC	CC
B-13	GC	SQTNCYRLS	CPC	KT	P	TQ	CP	VVI	CC
B-14	GC	SIEFLSFLG	CPC	LS	P	AF	CP	FLY	CC
B-15	GC	PEPLTWLTE	CPC	VE	P	IP	CP	DWM	CC
B-16	GC	LIMILFGEW	CPC	VM	P	LE	CP	PTR	CC
B-17	GC	LEFGCGVSS	CPC	EN	P	TH	CP	WAH	CC
B-18	GC	ARESGFMIL	CPC	QS	P	RA	CP	ESS	CC
B-19	GC	FLFFSRLPE	CPC	SS	P	CF	CP	PDR	CC
B-20	GC	YAVVCLHHE	CPC	LY	P	LL	CP	CLW	CC

Figure S16. Sequences obtained from screening of **8-X₁₆** against Bcl-2 after four rounds of panning.

2.6 Binding affinity of the oxidized peptides to target proteins

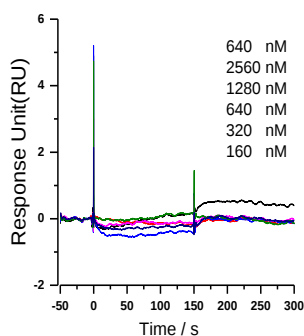


Figure S17. Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of **16-Ala** with Bcl-2.

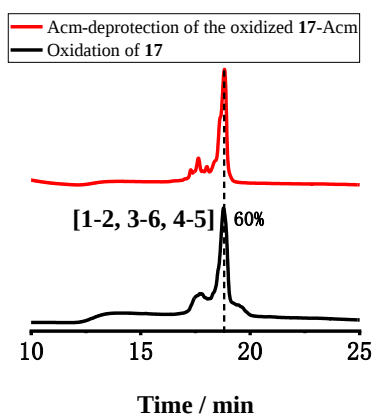


Figure S18. Chromatogram of products formed after the oxidation of **17** (black line) and the Acm-deprotection of the oxidized **17-Acm** (red line).

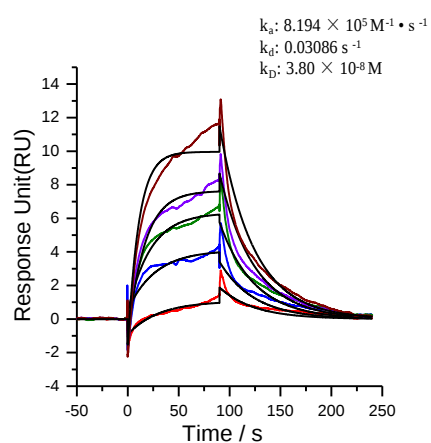


Figure S19. Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized **17** with streptavidin.

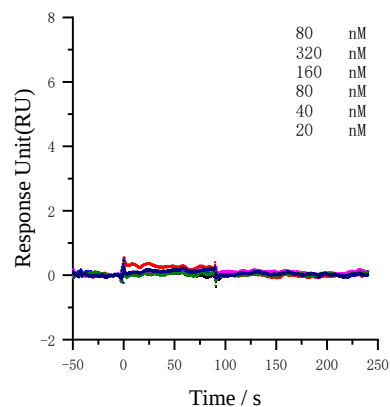
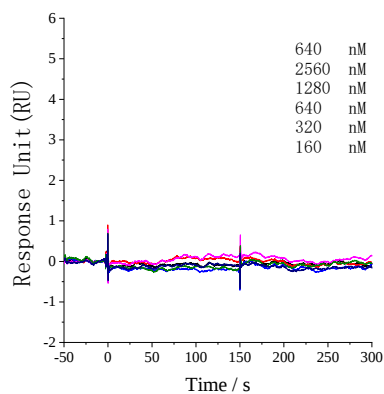


Figure S20. Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized **11** with streptavidin.

a)



b)

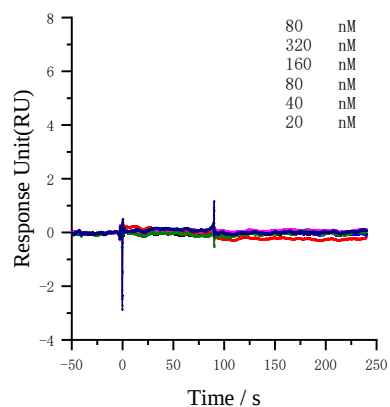
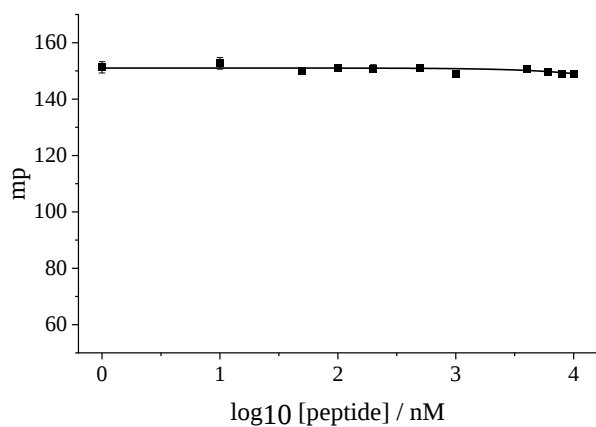


Figure S21. a) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of oxidized **15** with Bcl-2. b) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized **15** with streptavidin.

a)



b)

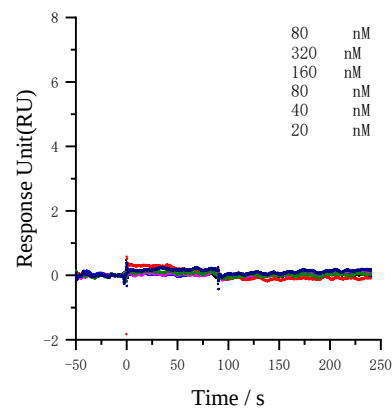
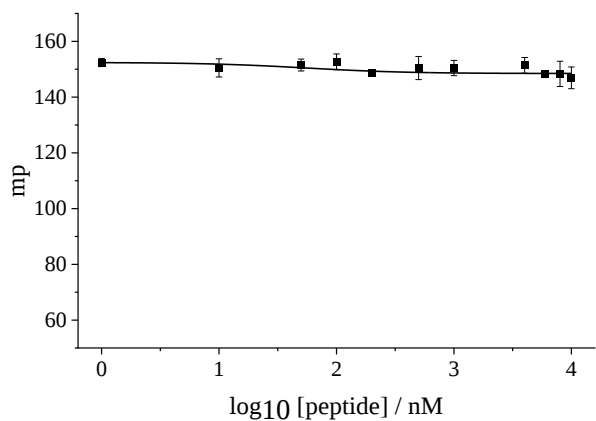


Figure S22. a) A fluorescence polarization competition assay showing the binding of oxidized **16** to MDM2. b) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized **16** with streptavidin.

a)



b)

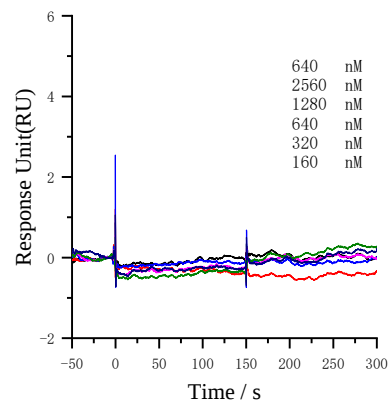
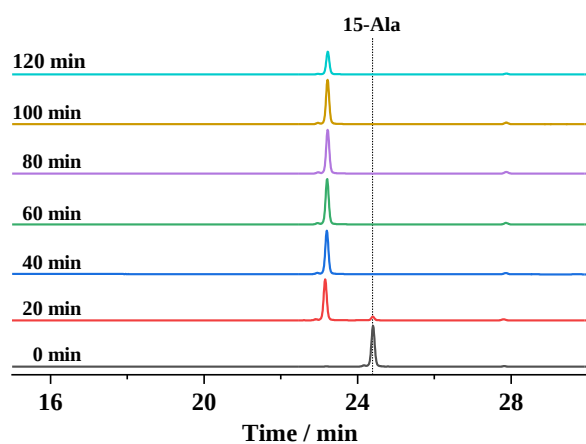


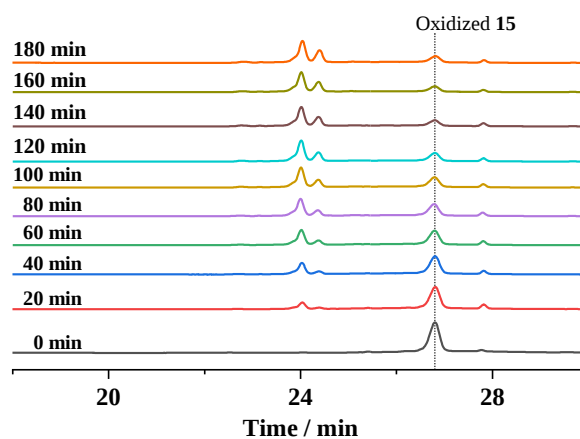
Figure S23. a) A fluorescence polarization competition assay showing the binding of oxidized **17** to MDM2. b) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized **17** with Bcl-2.

2.7 Proteolytic stability of peptides

a)



b)



c)

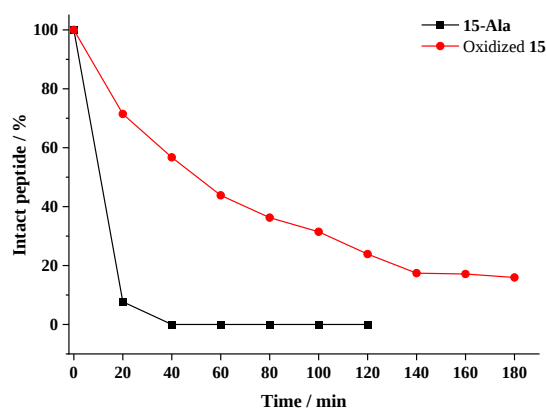


Figure S24. a) Chromatograms of 15-Ala digested by chymotrypsin. b) Chromatograms of oxidized 15 digested by chymotrypsin. c) Kinetics of peptide degradation by chymotrypsin in phosphate buffer at pH 7.4. Concentrations of peptides and chymotrypsin were 100 μM and 300 $\text{ng}\cdot\text{mL}^{-1}$, respectively.

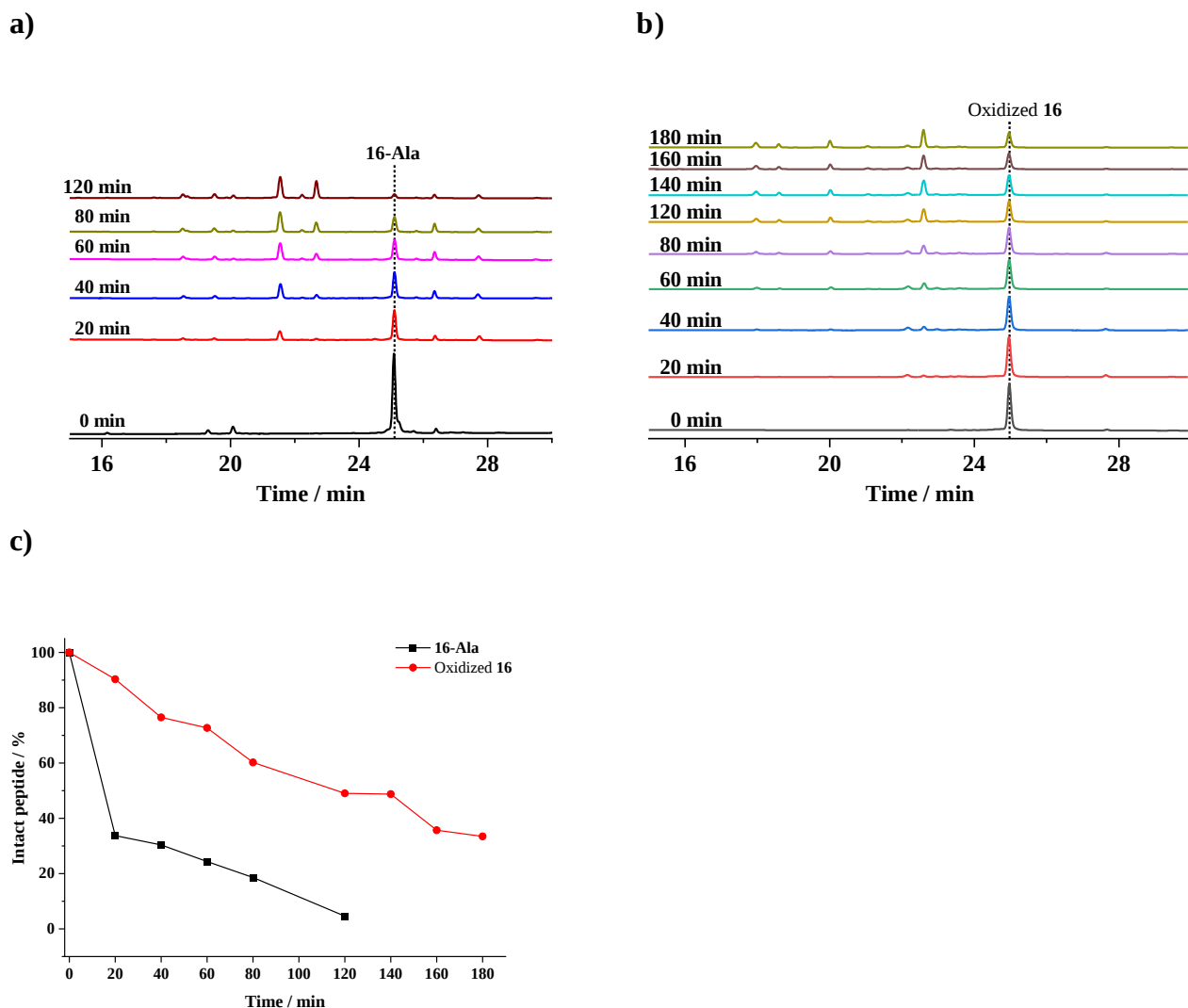
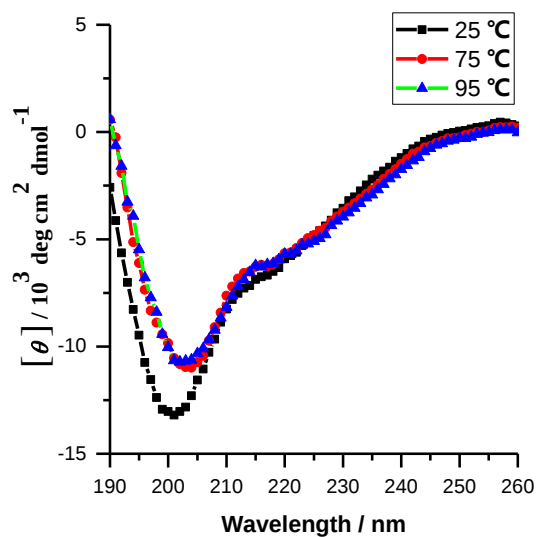


Figure S25. a) Chromatograms of **16**–Ala digested by chymotrypsin. b) Chromatograms of oxidized **16** digested by chymotrypsin. c) Kinetics of peptide degradation by chymotrypsin in phosphate buffer at pH 7.4. Concentrations of peptides and chymotrypsin were 100 μM and 5 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively.

2.8 CD spectra of the oxidized **15** and oxidized **16**

a)



b)

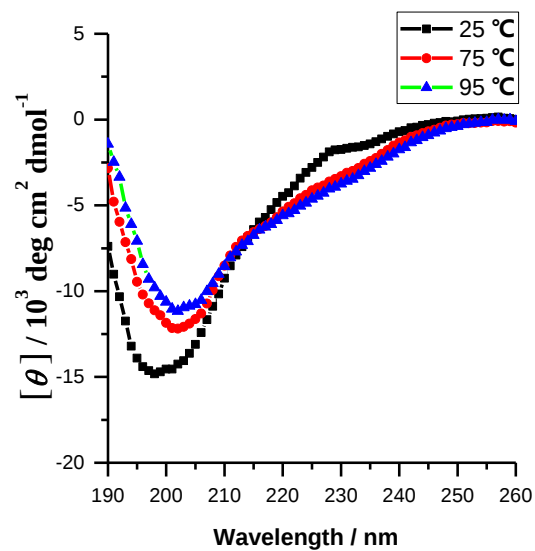
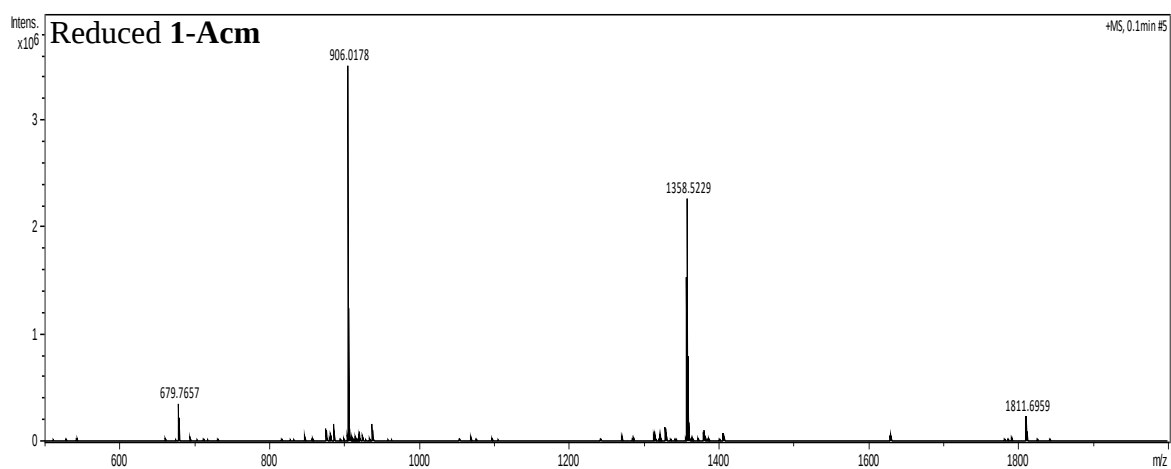
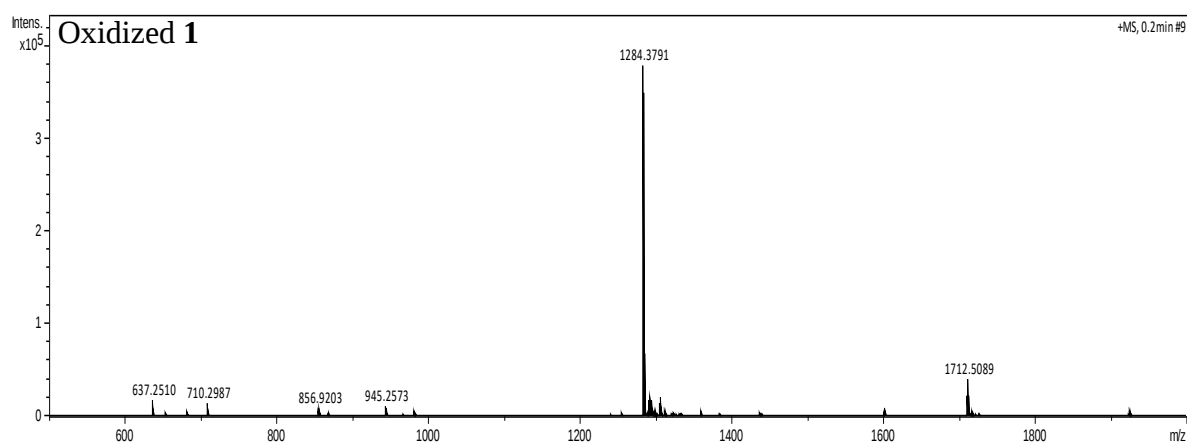
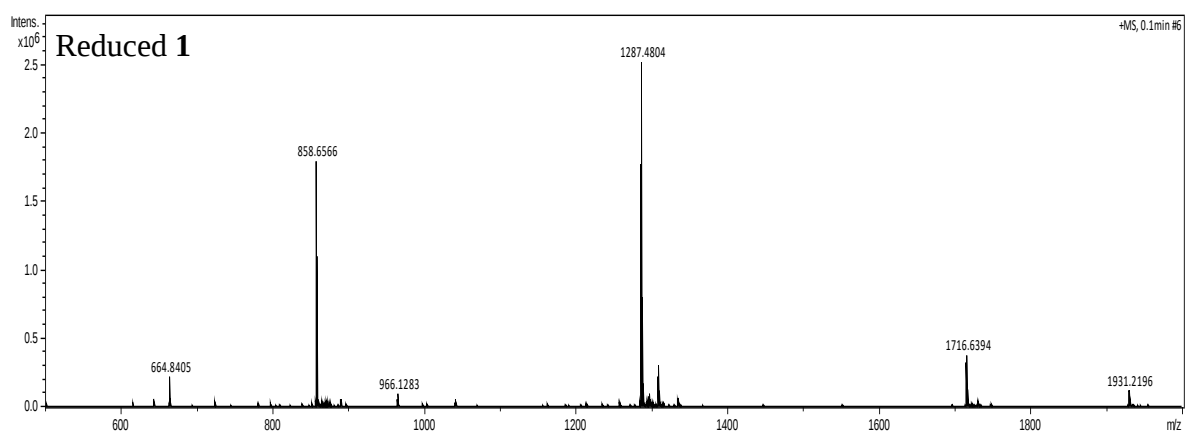
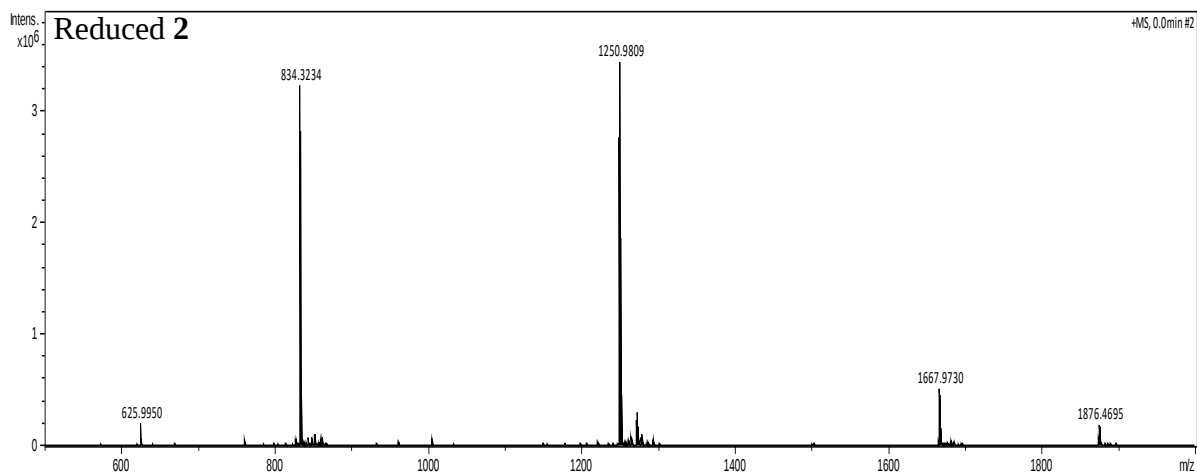
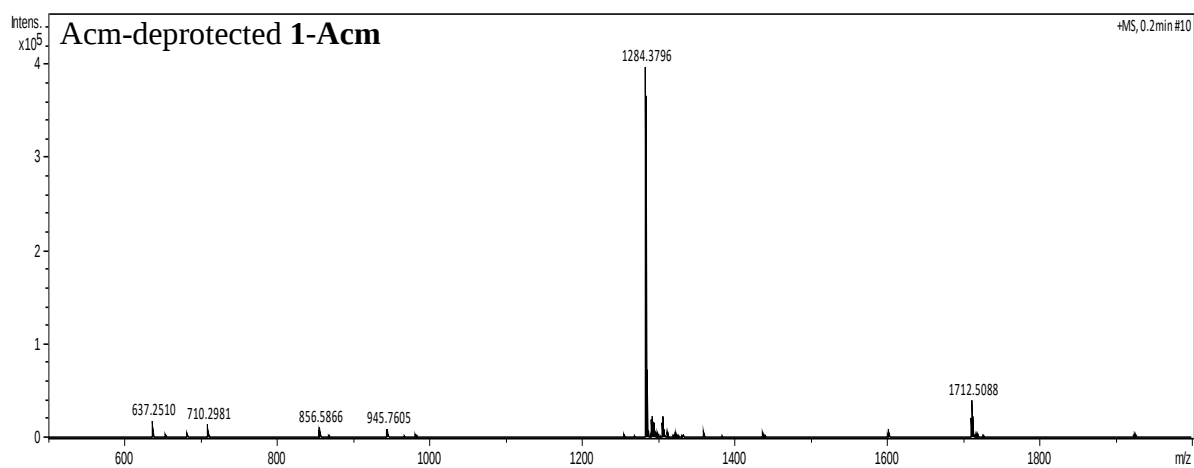
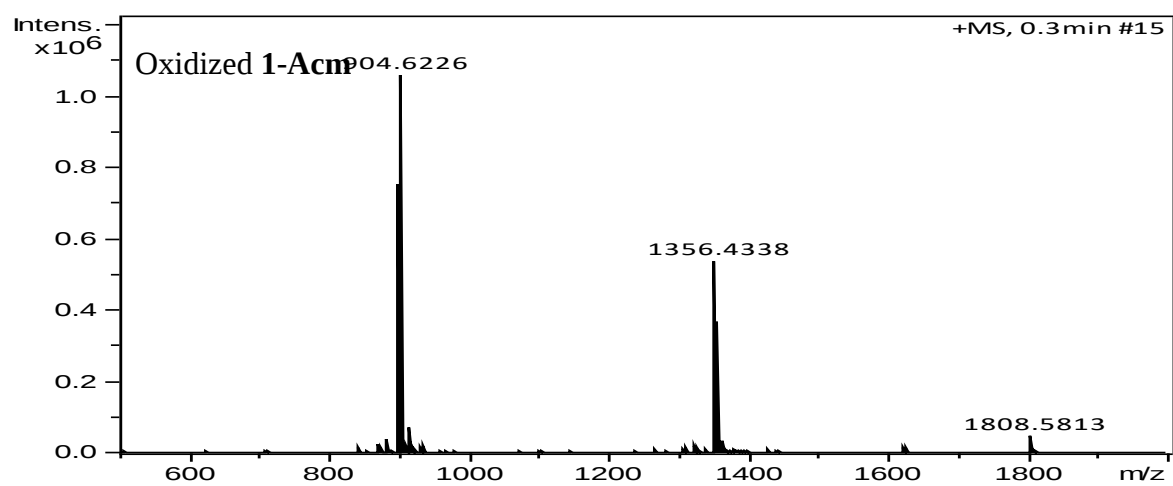
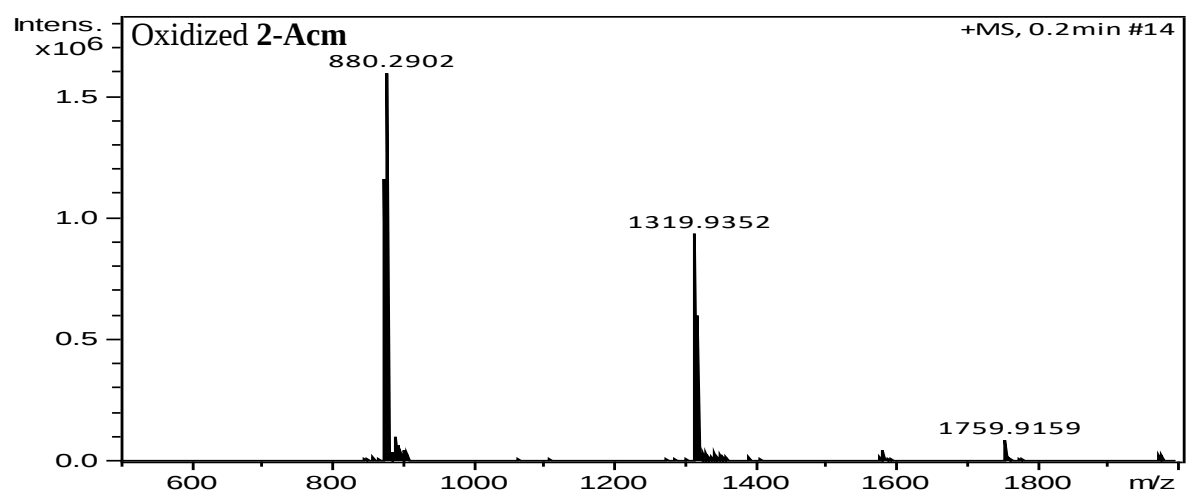
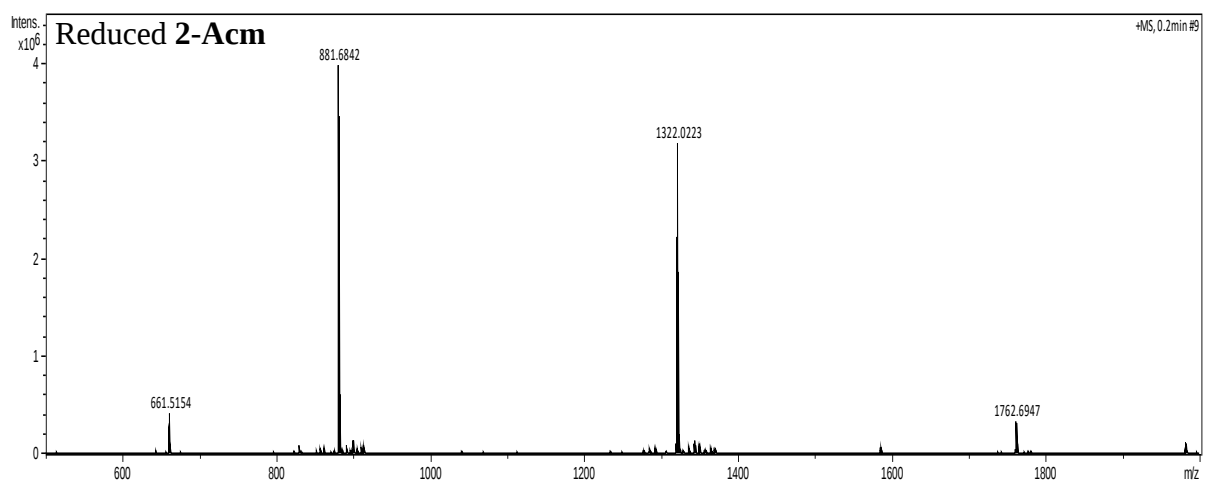
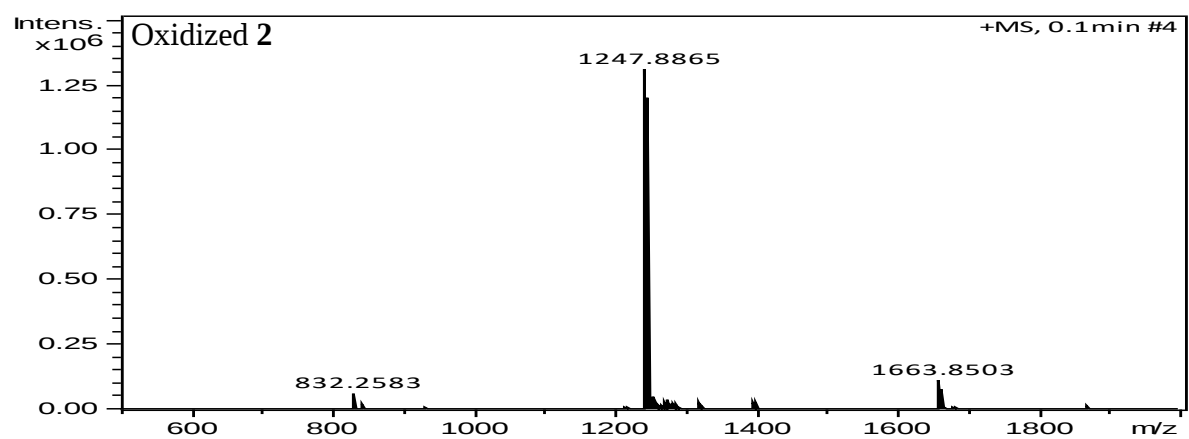


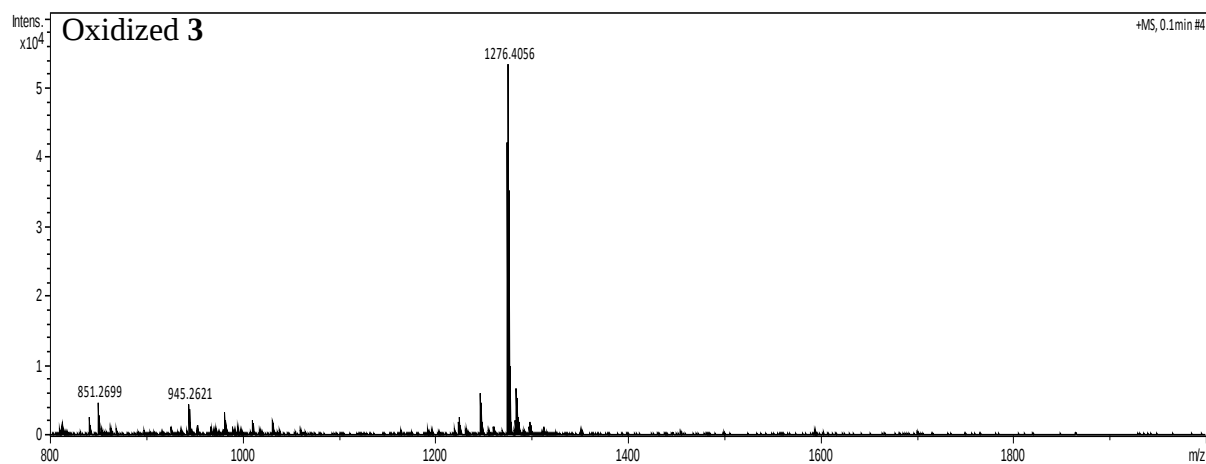
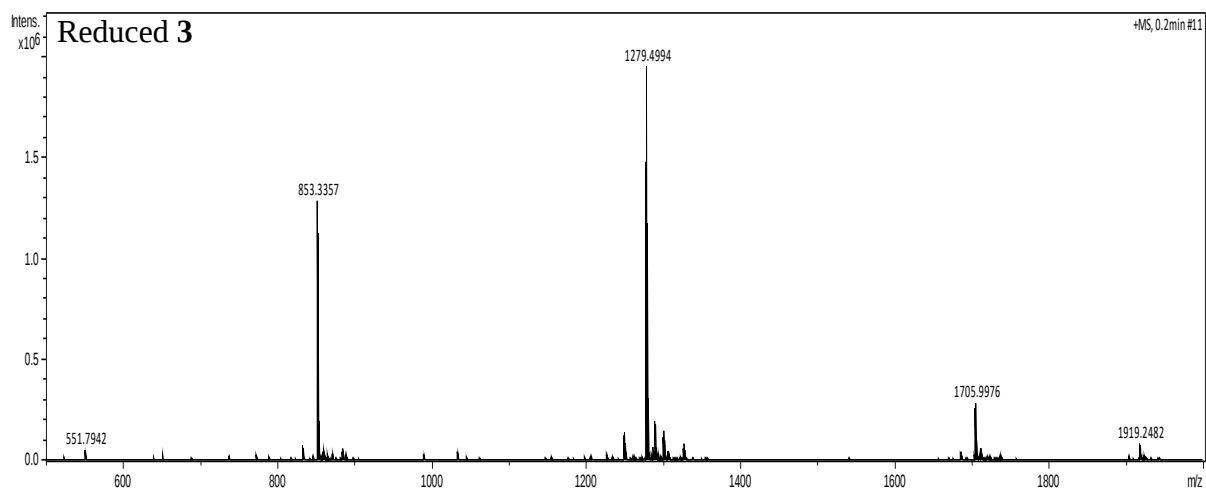
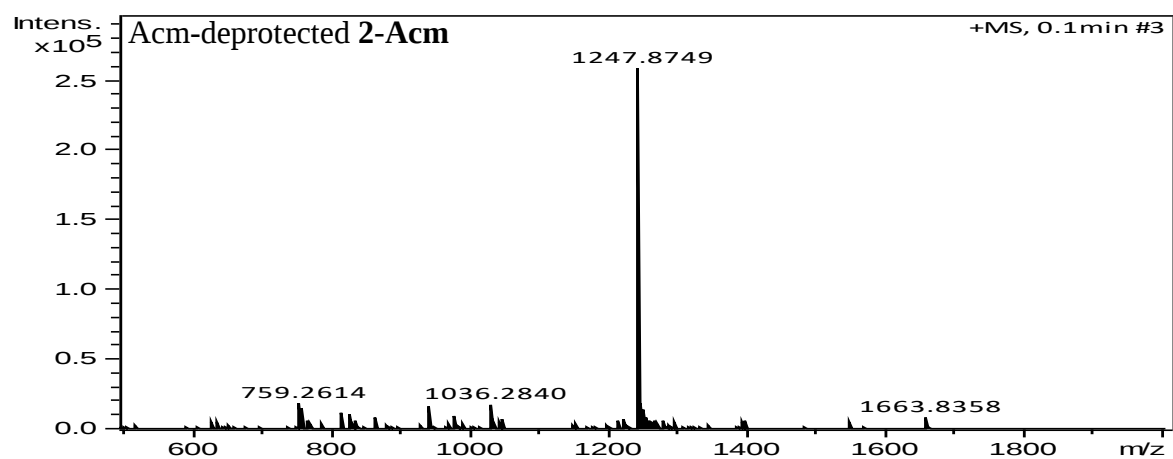
Figure S26. a) CD spectra of the oxidized **15** (50 μM) in water. b) CD spectra of the oxidized **16** (50 μM) in water.

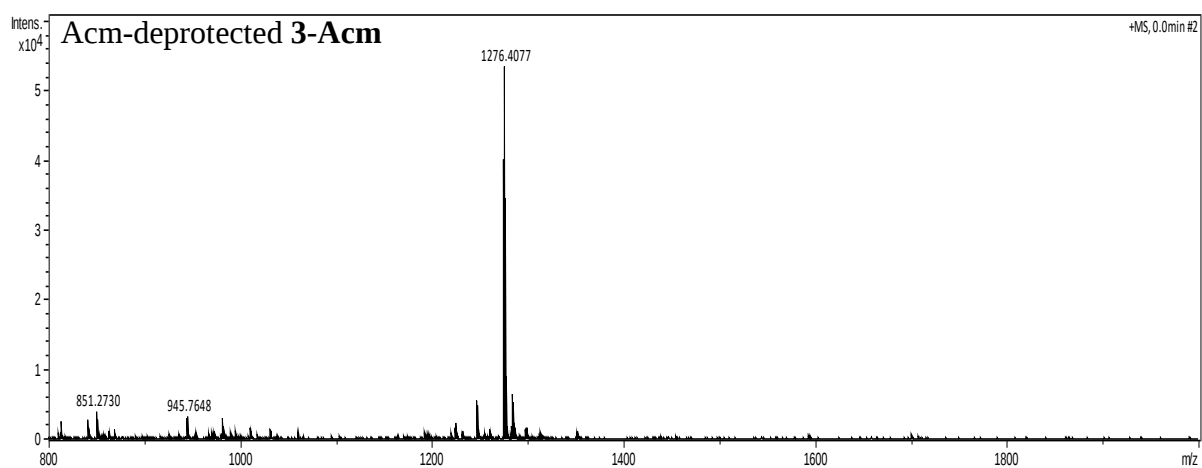
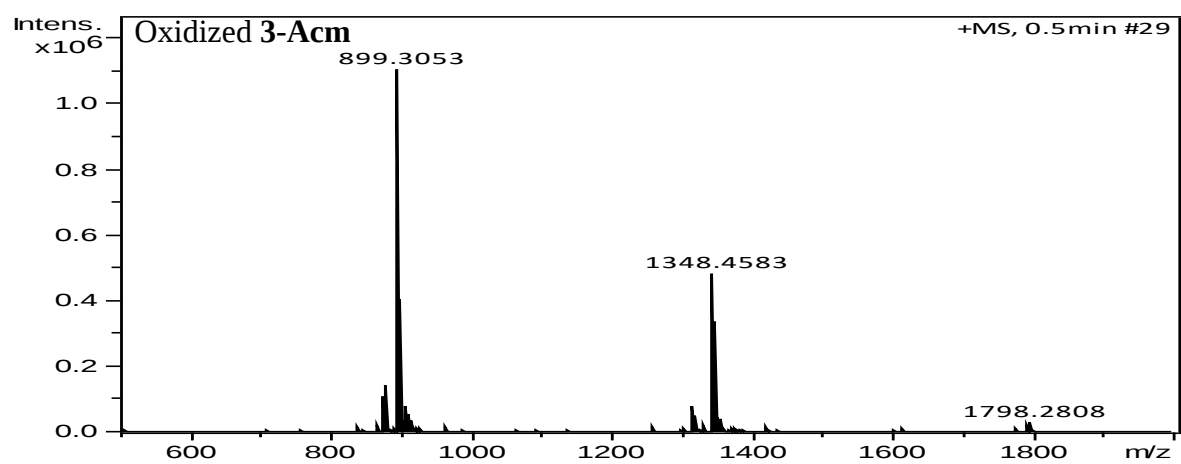
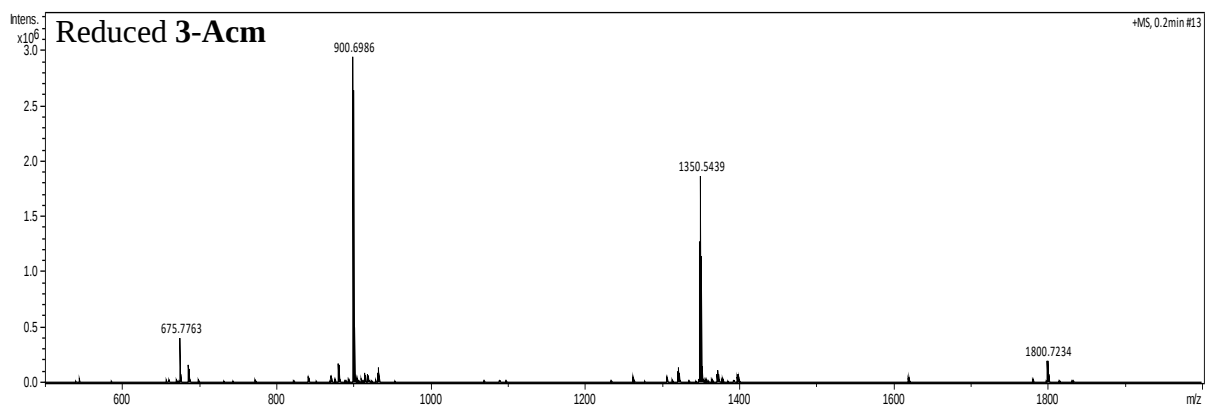
2.9 Mass spectra of peptides

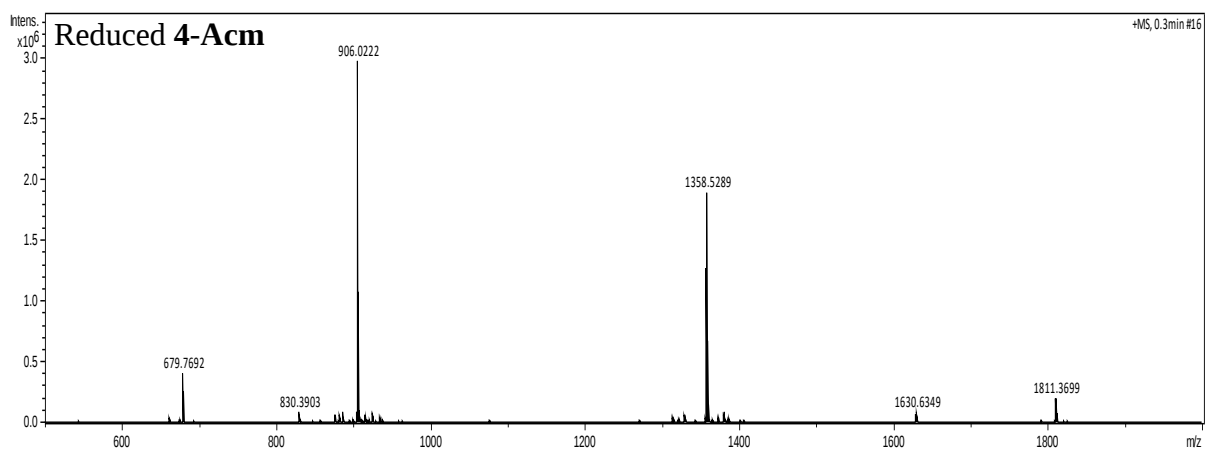
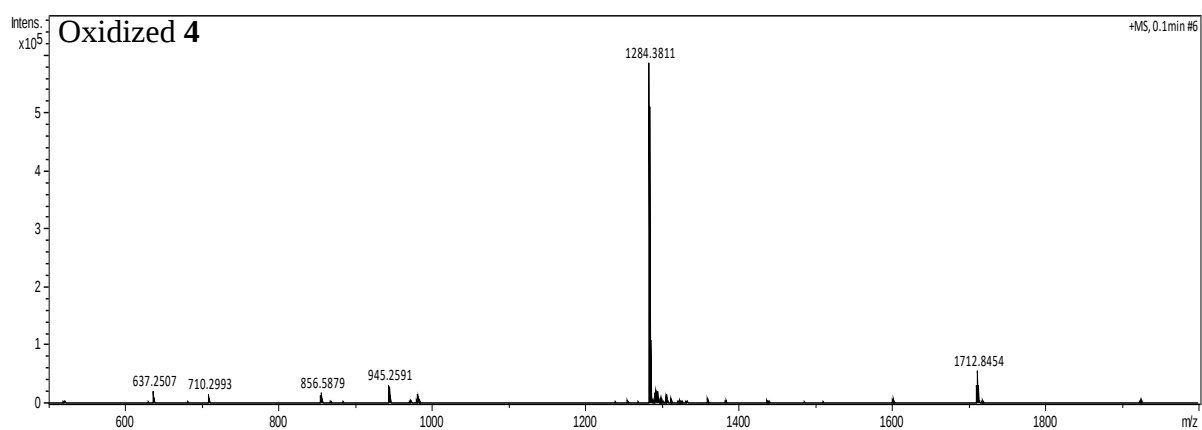
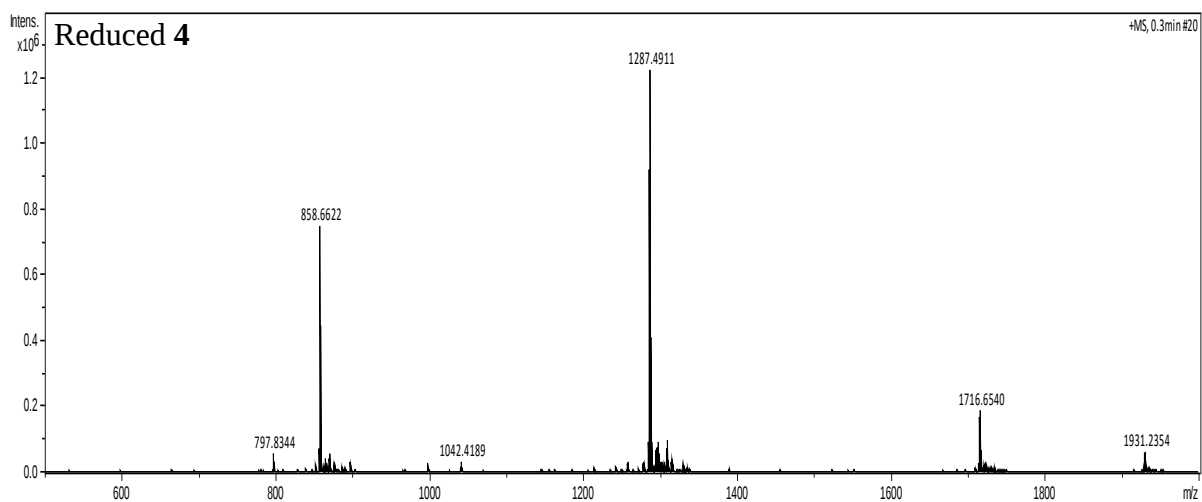


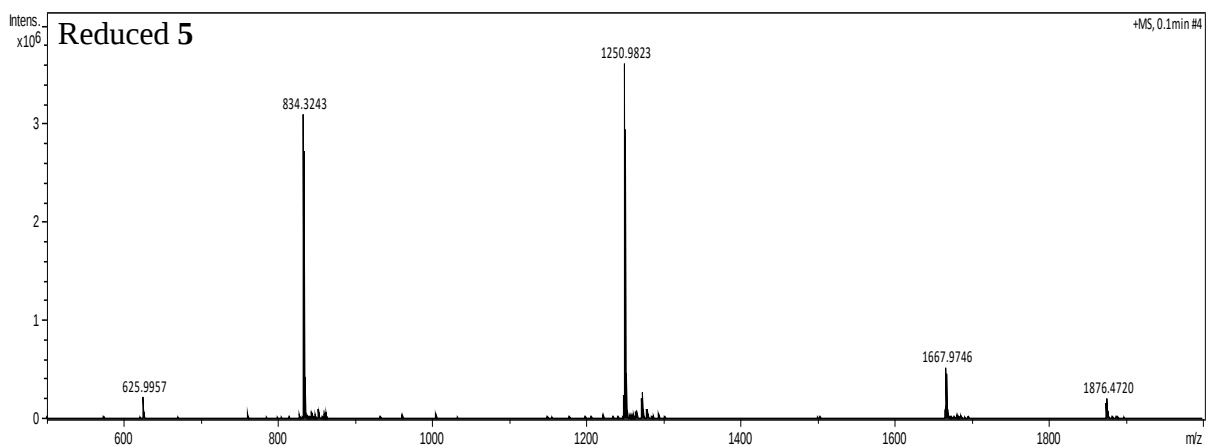
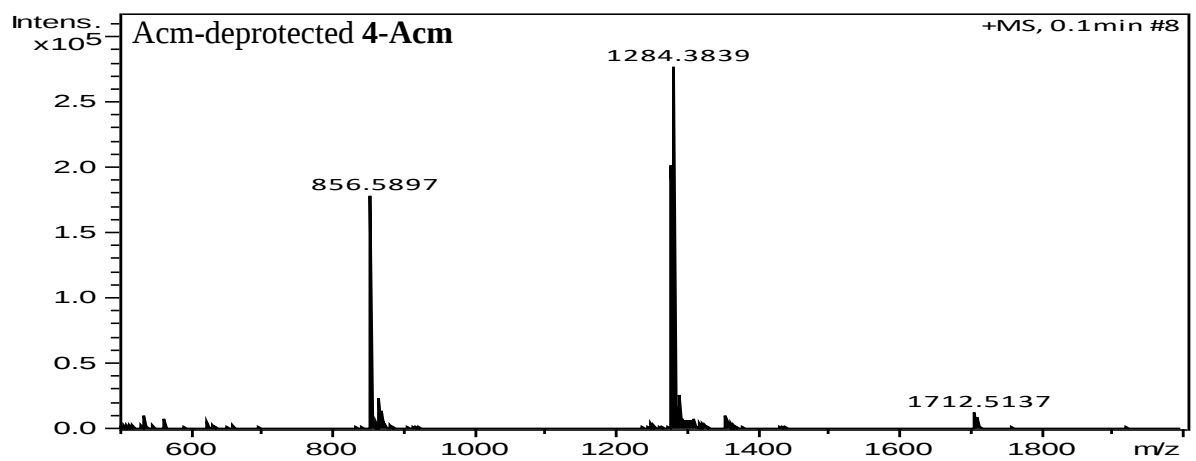
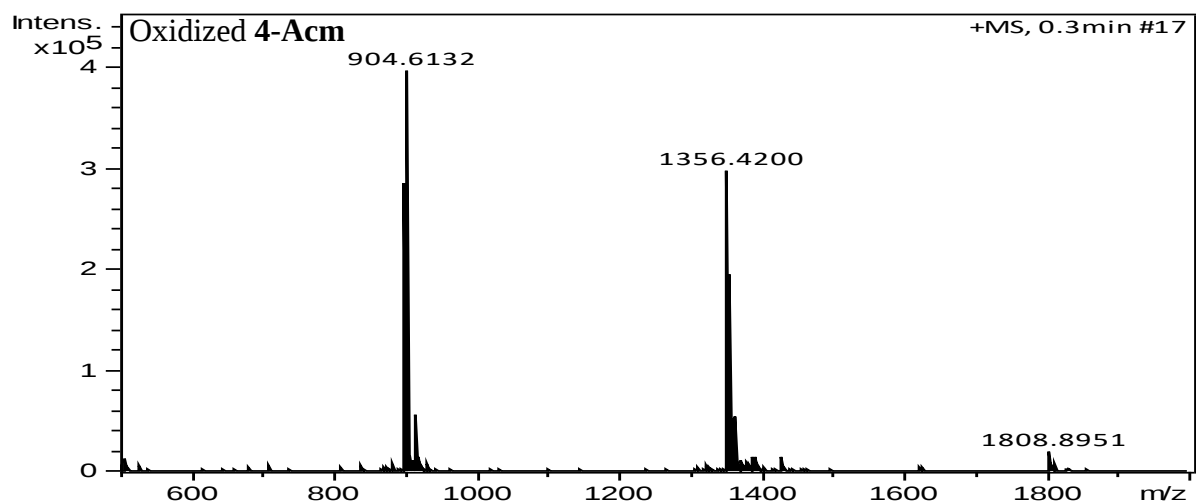


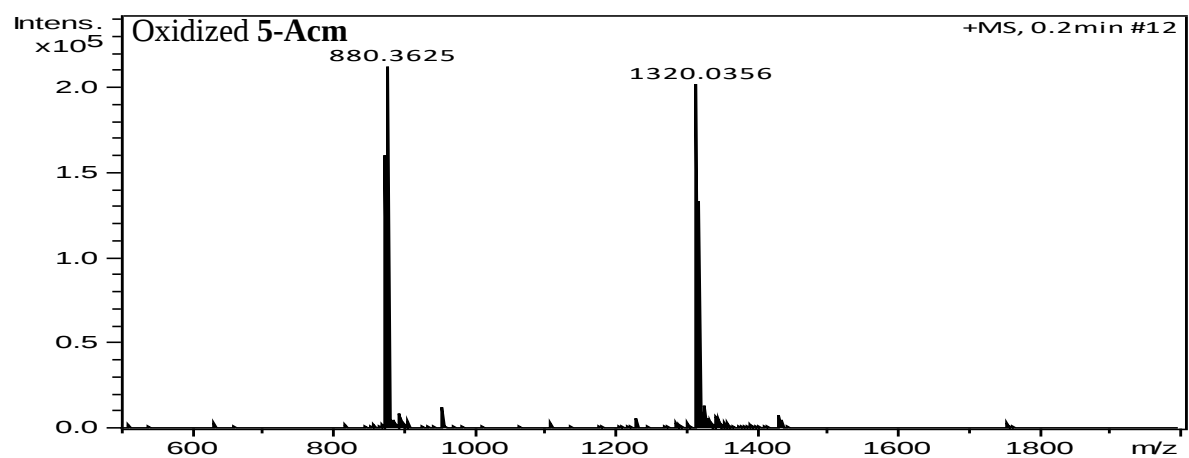
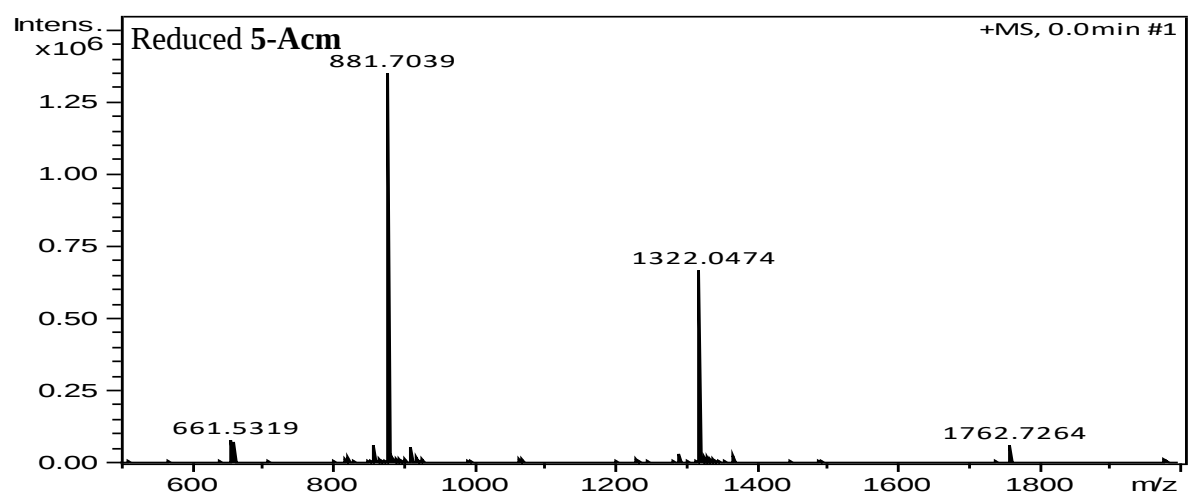
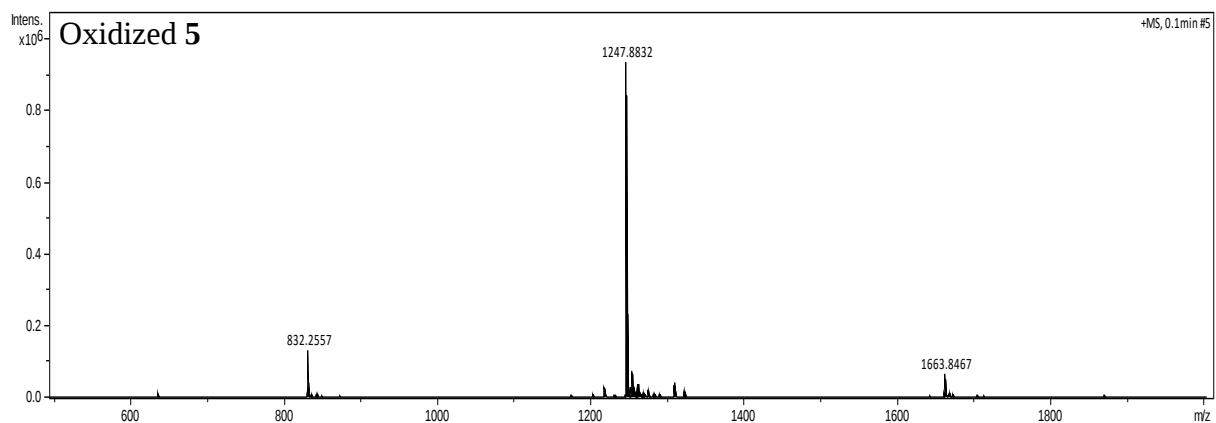


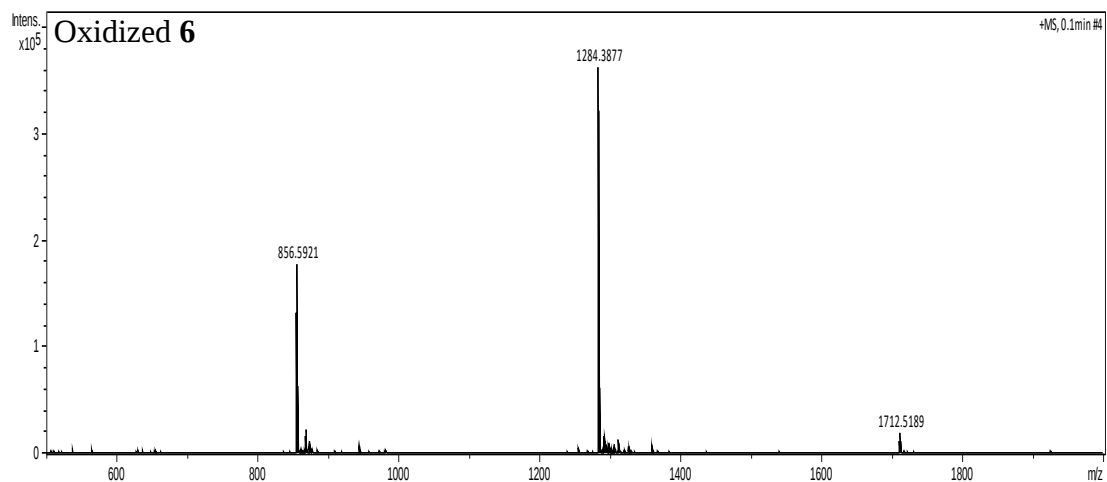
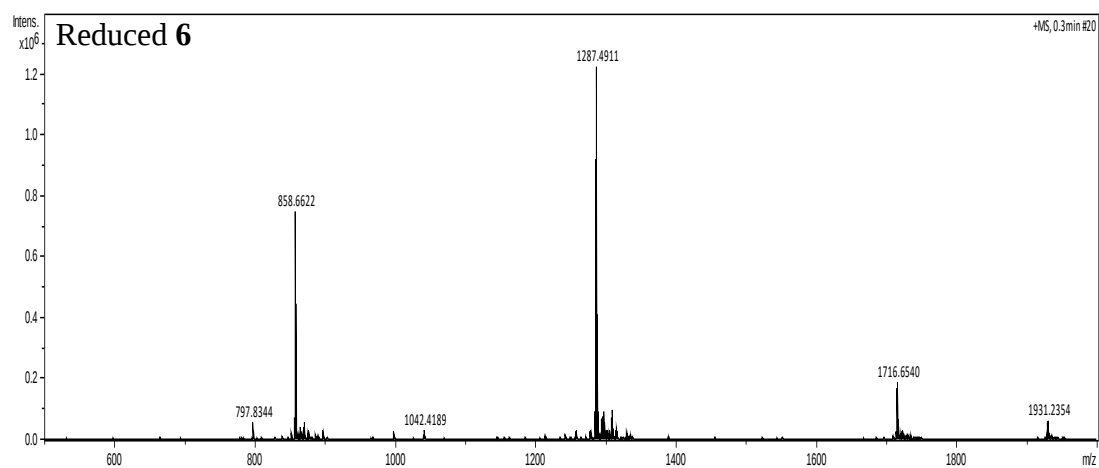
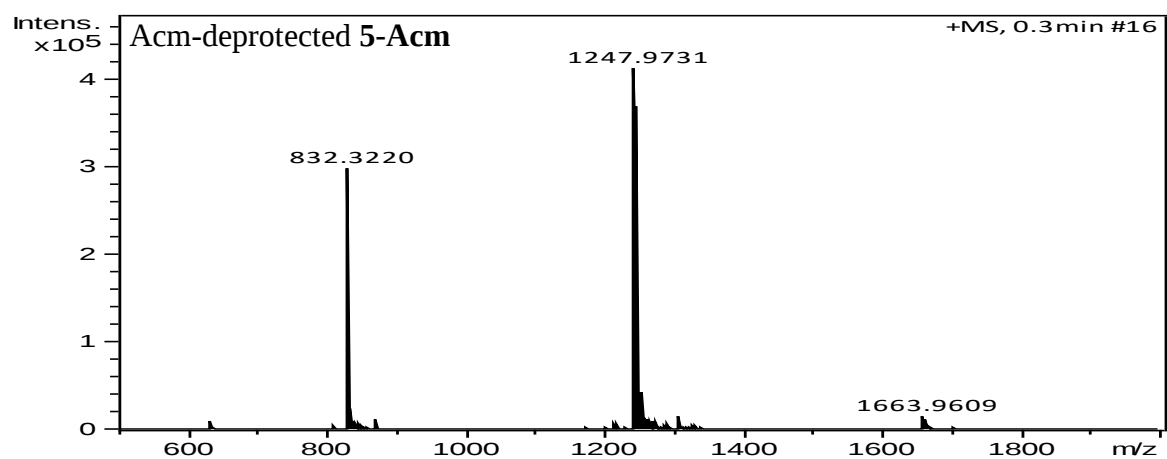


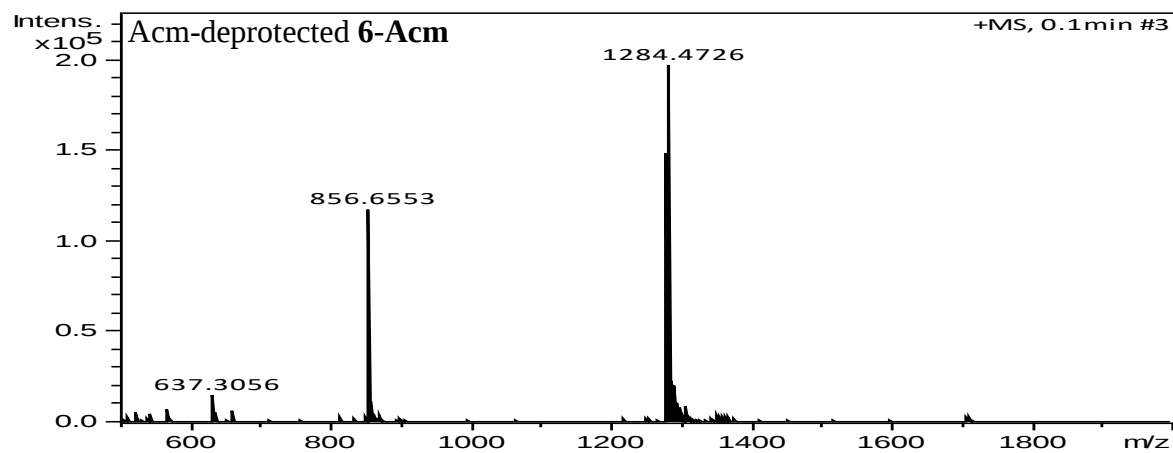
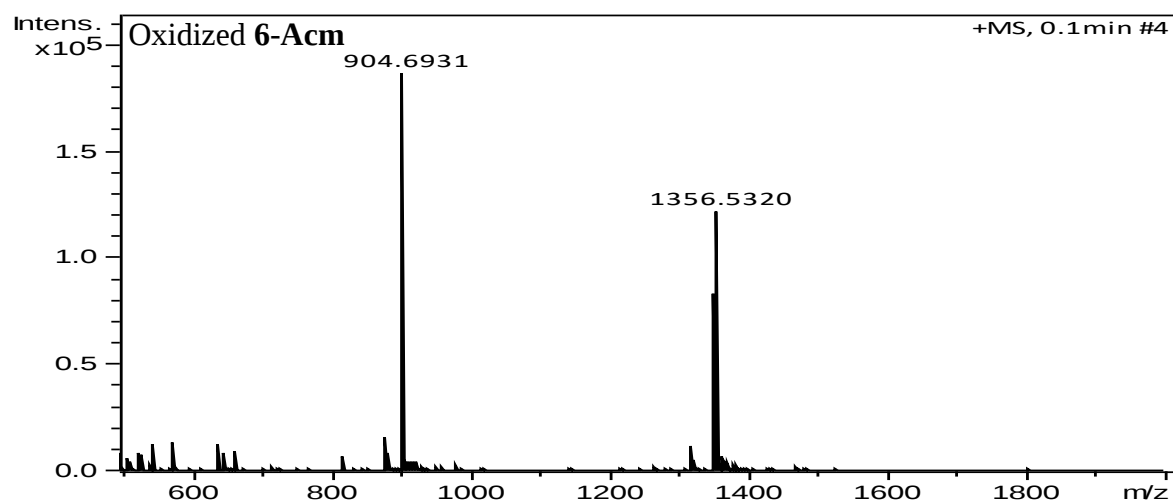
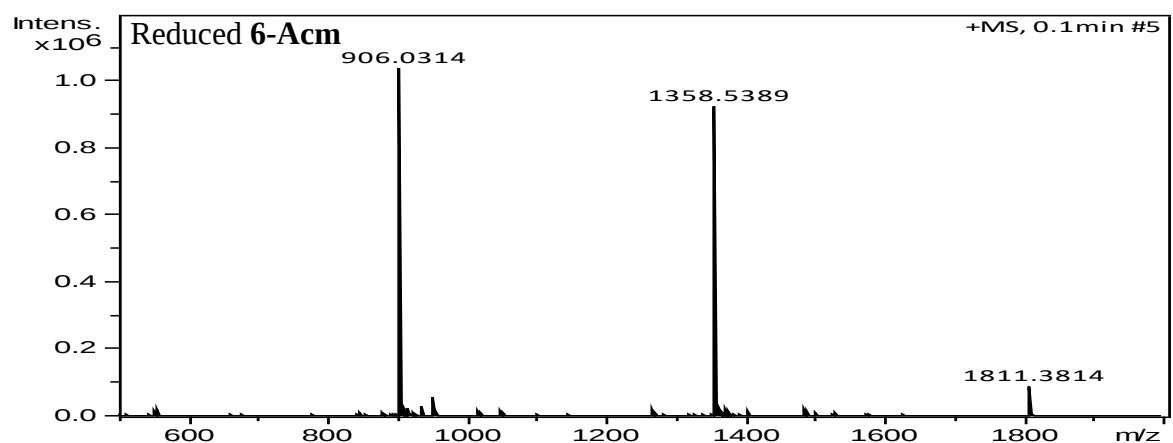


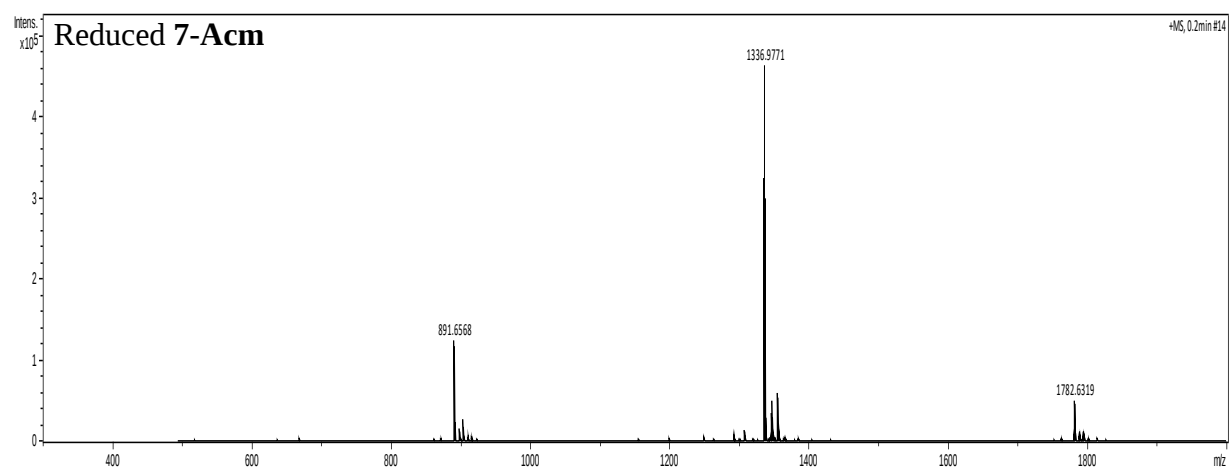
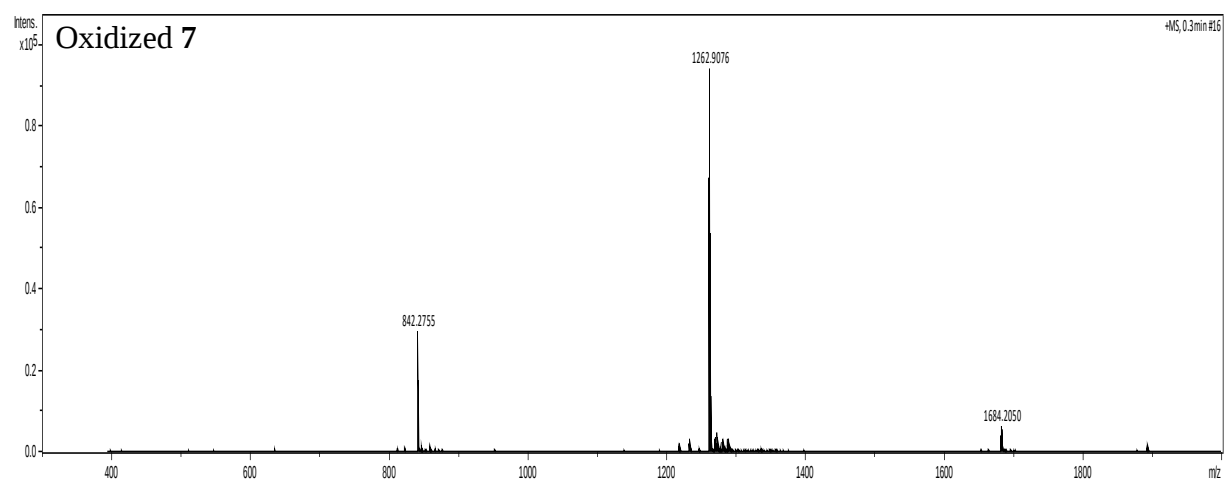
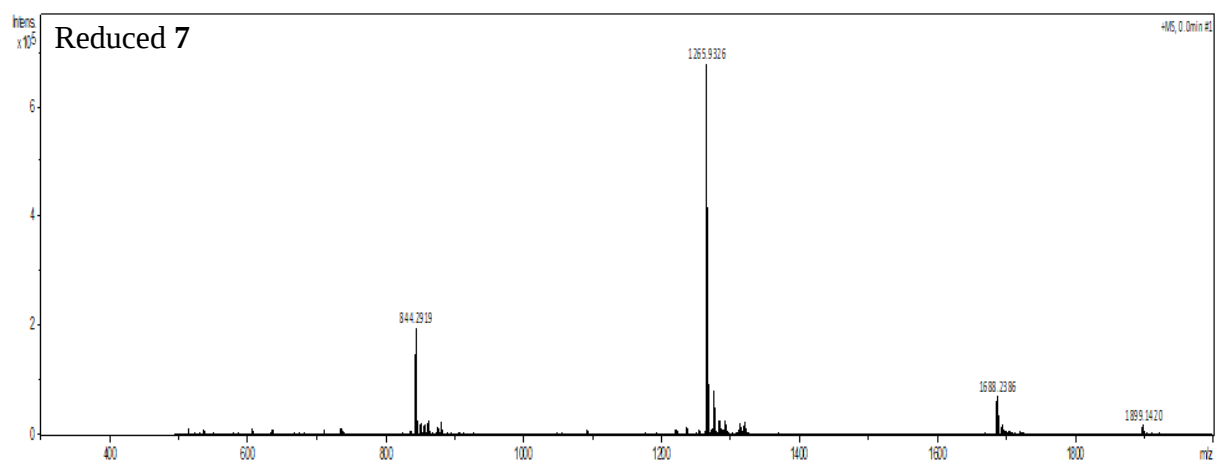


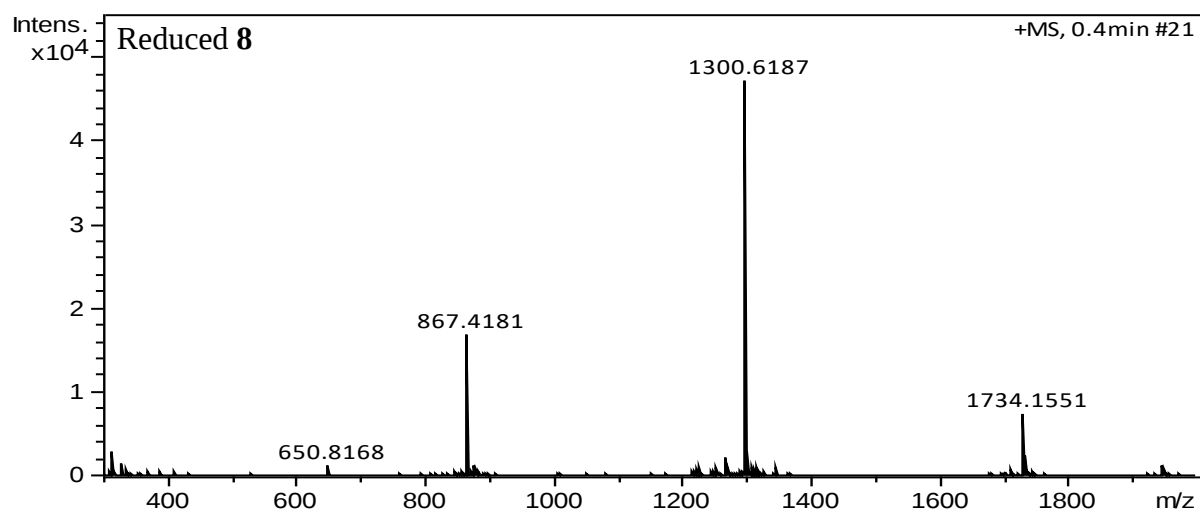
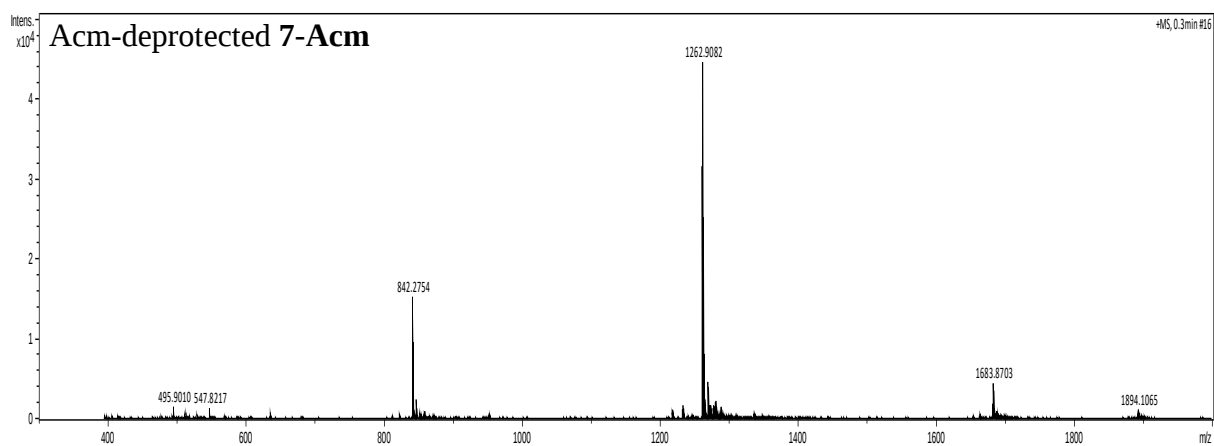
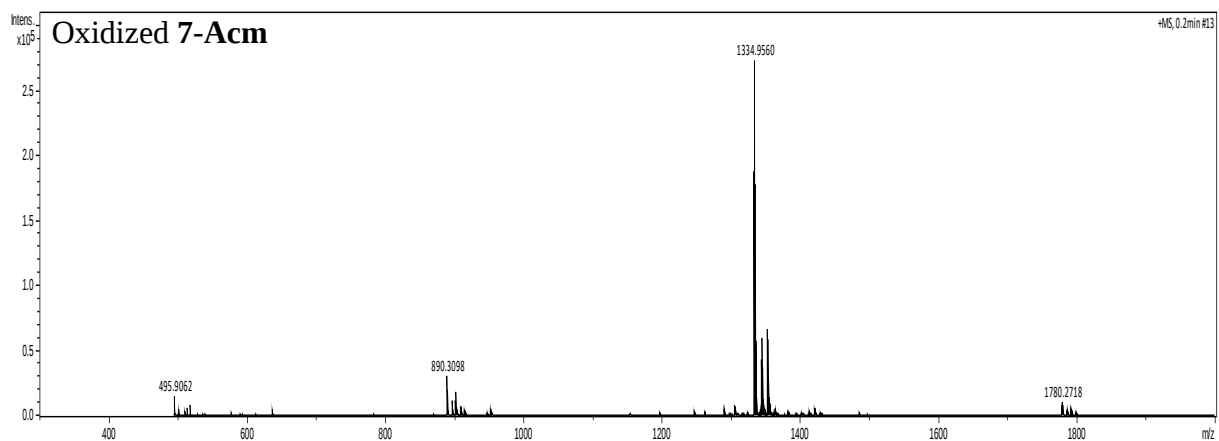


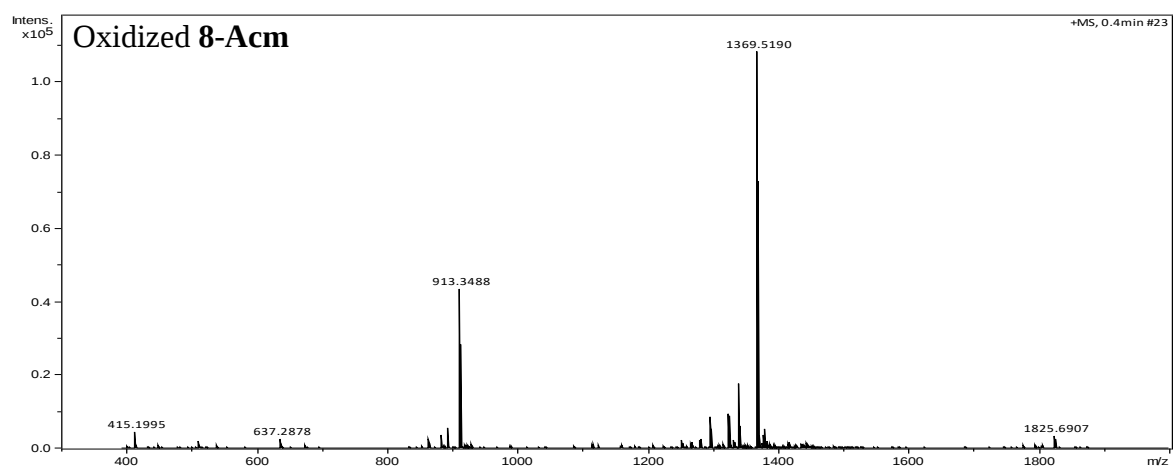
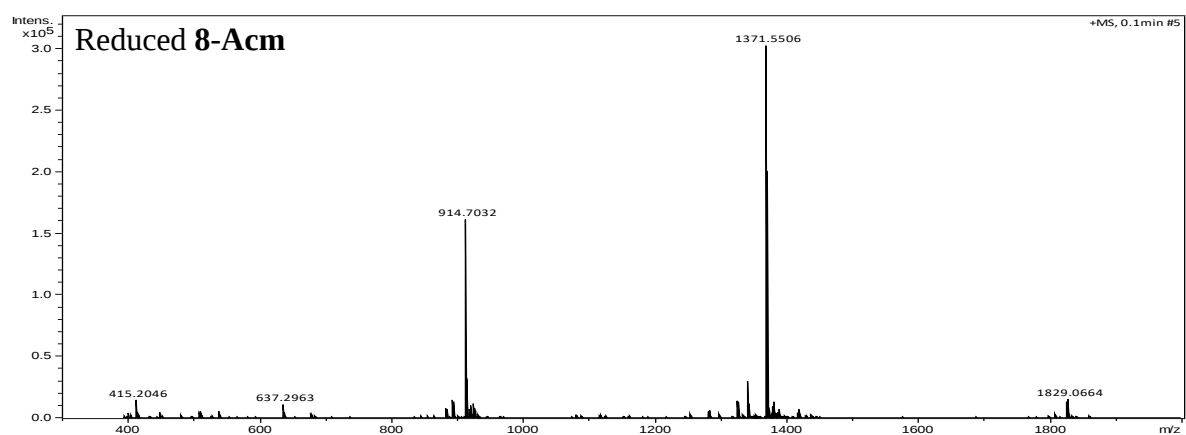
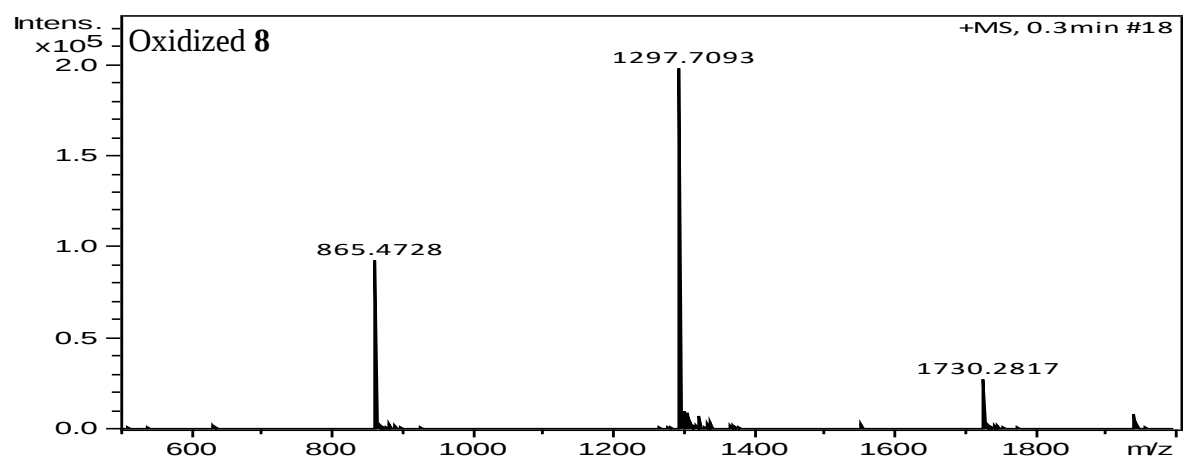


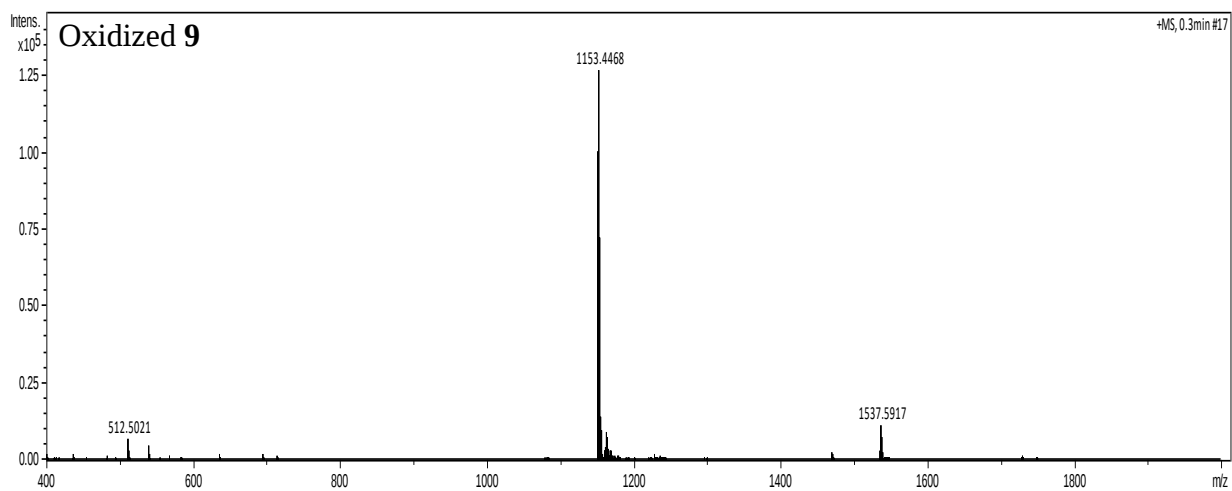
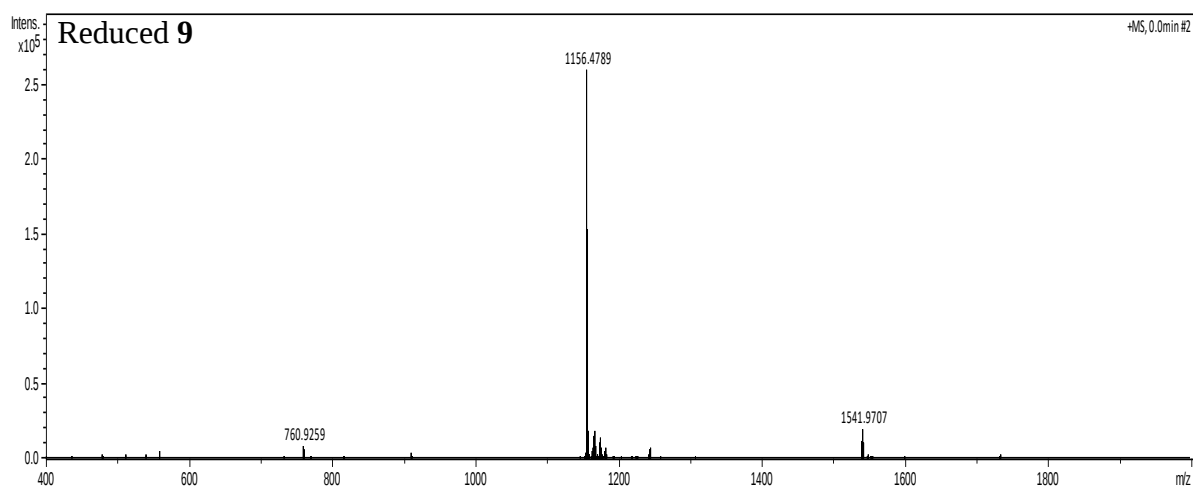
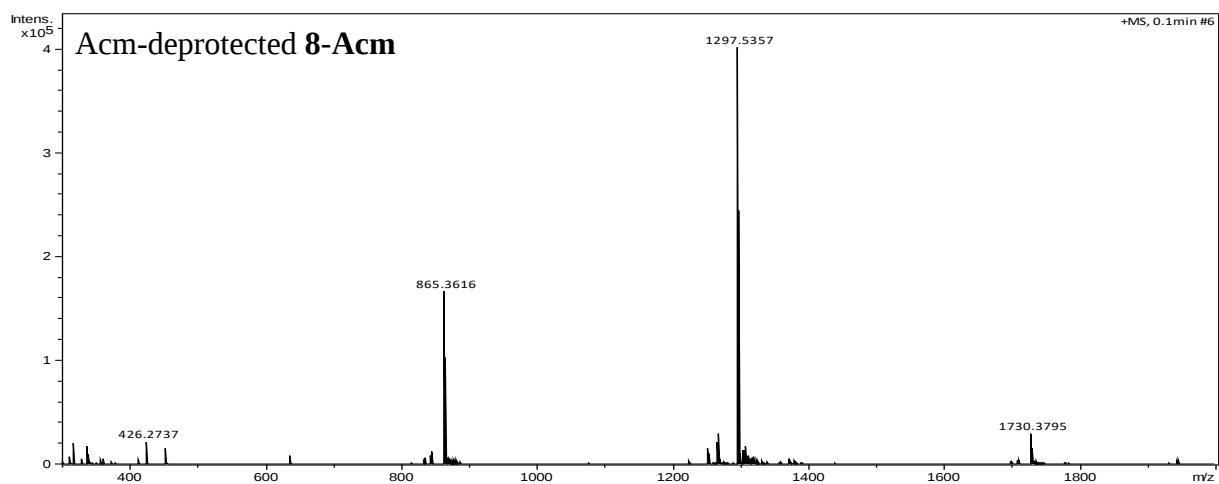


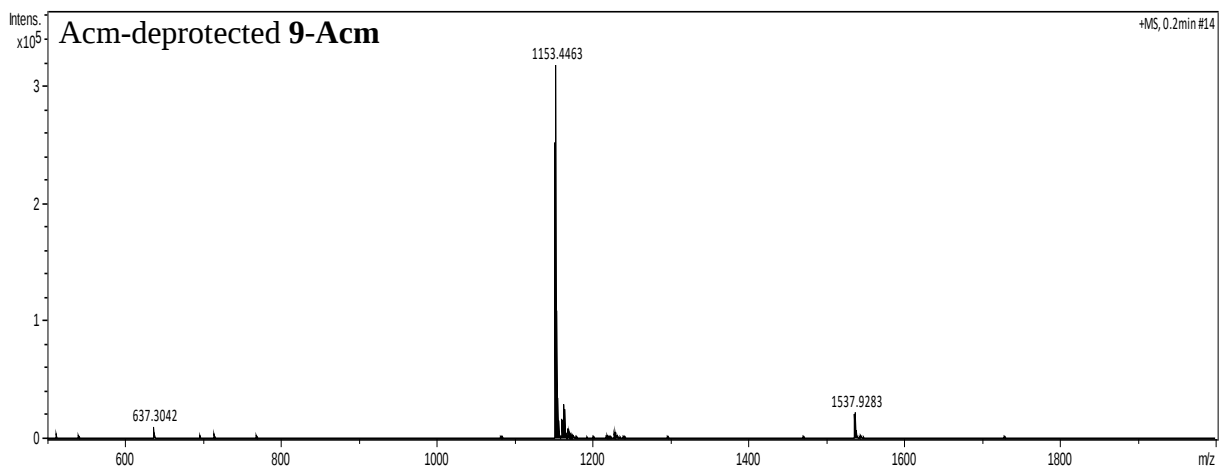
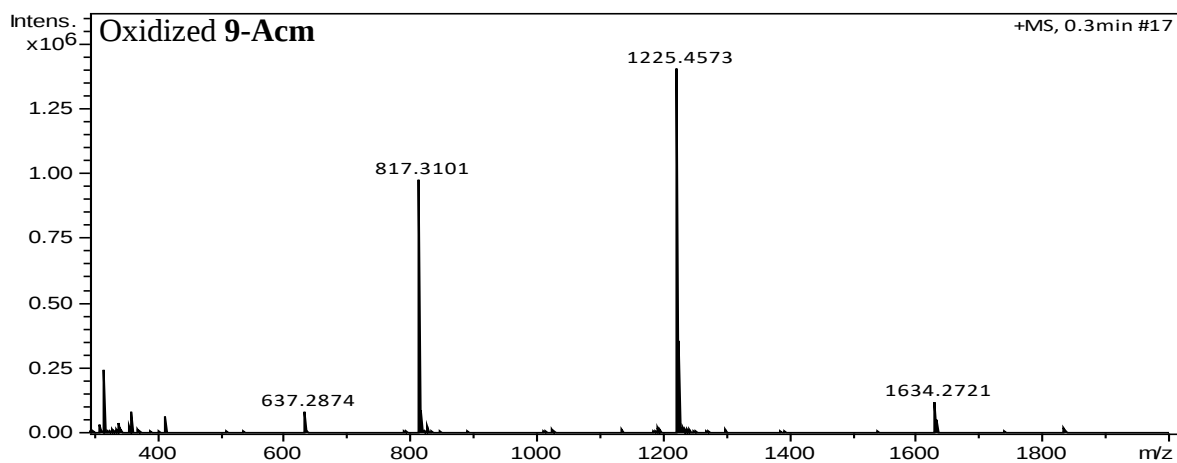
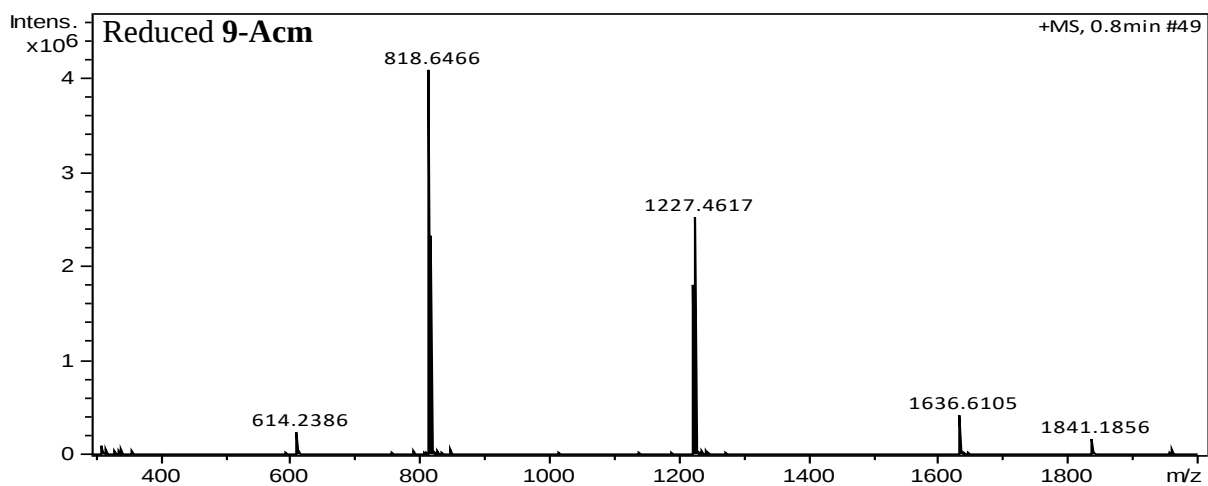


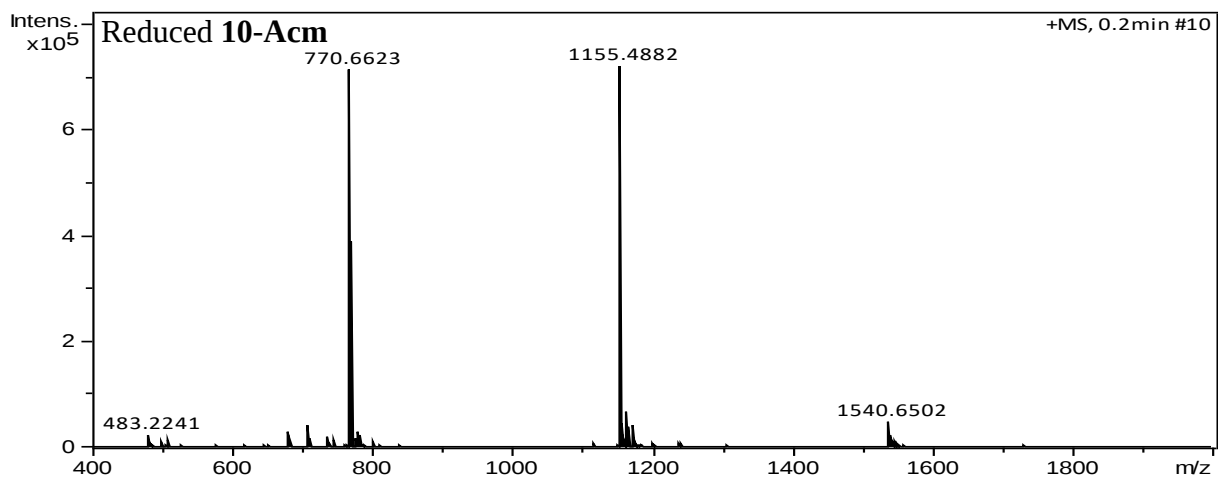
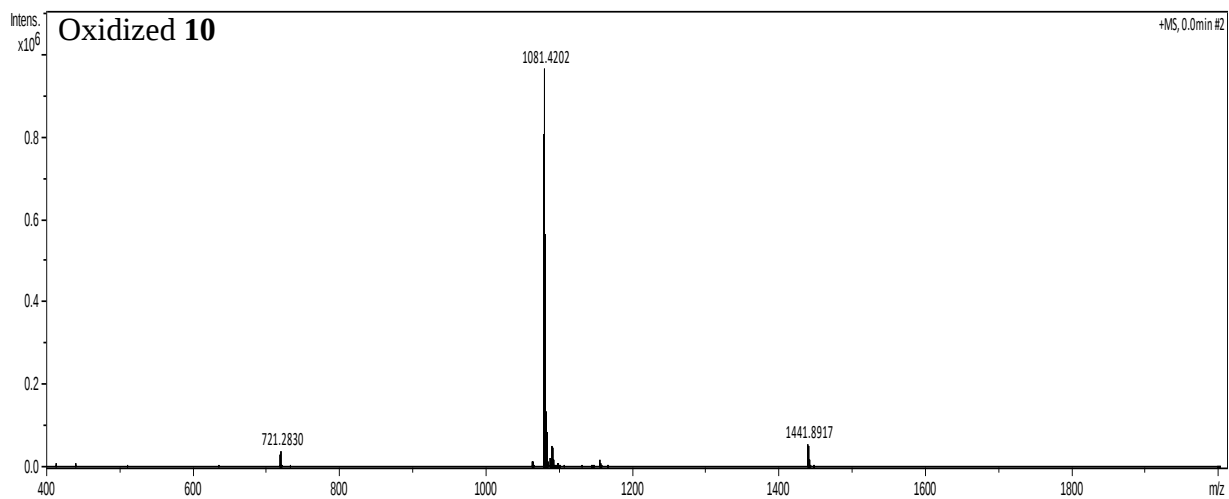
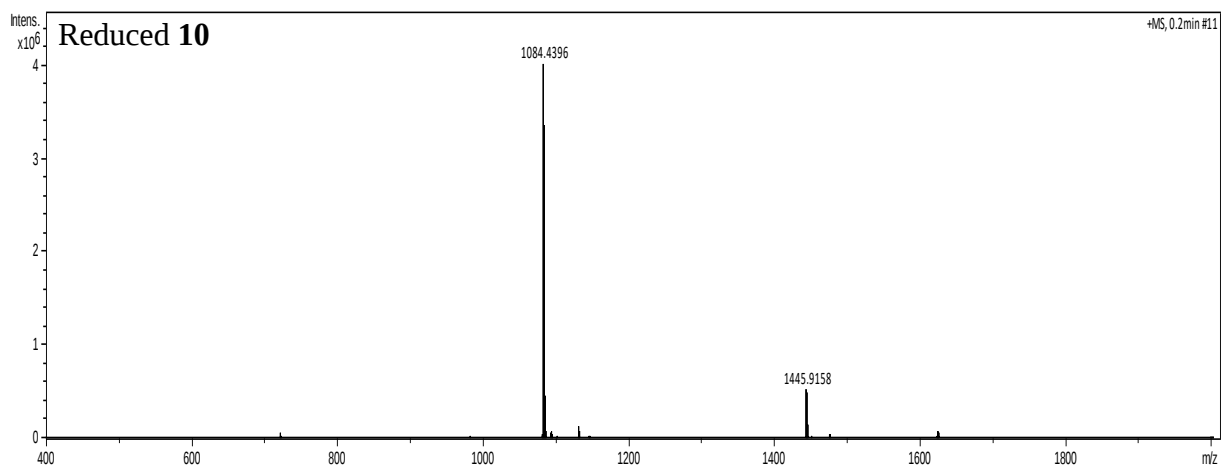


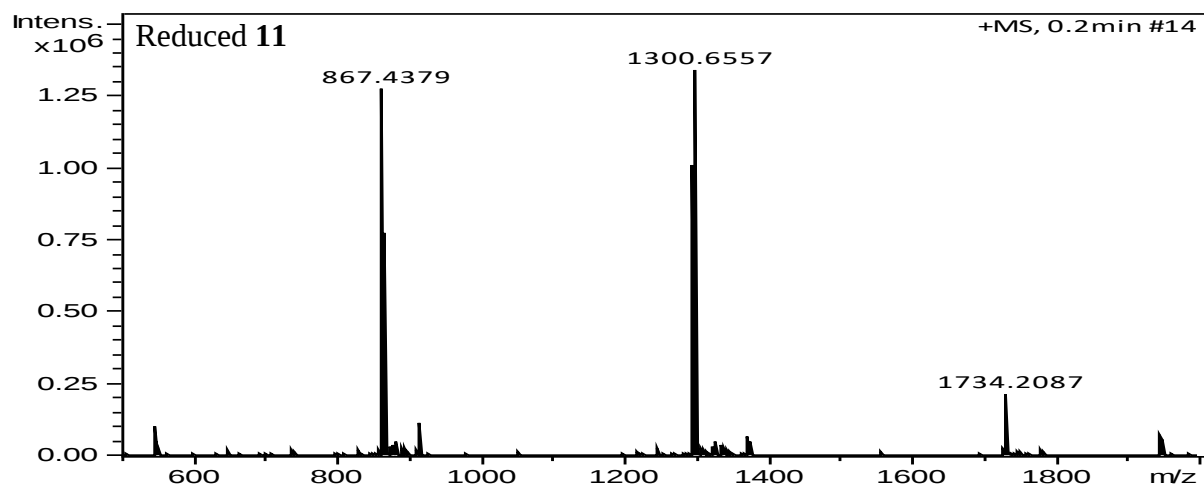
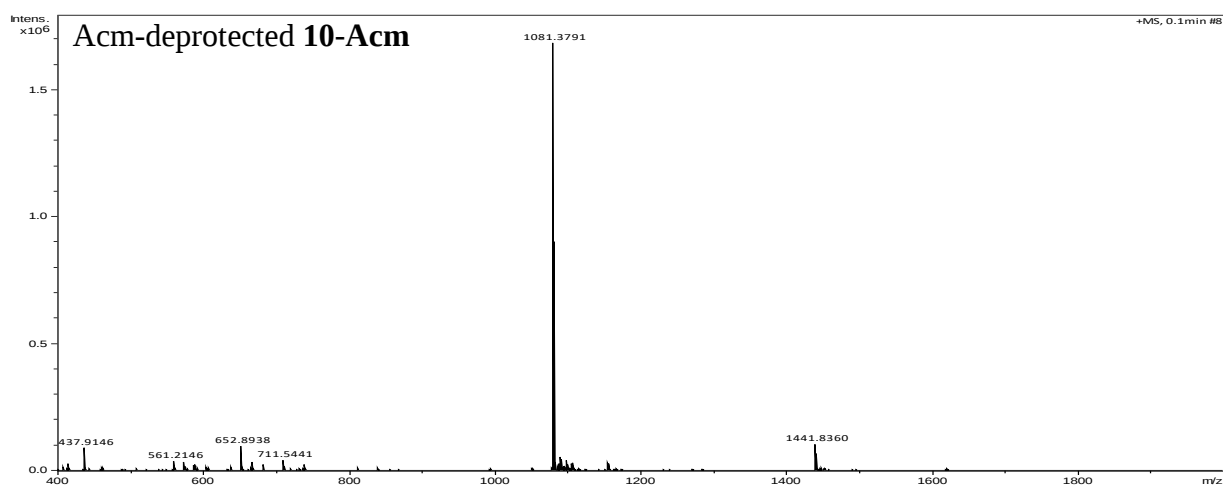
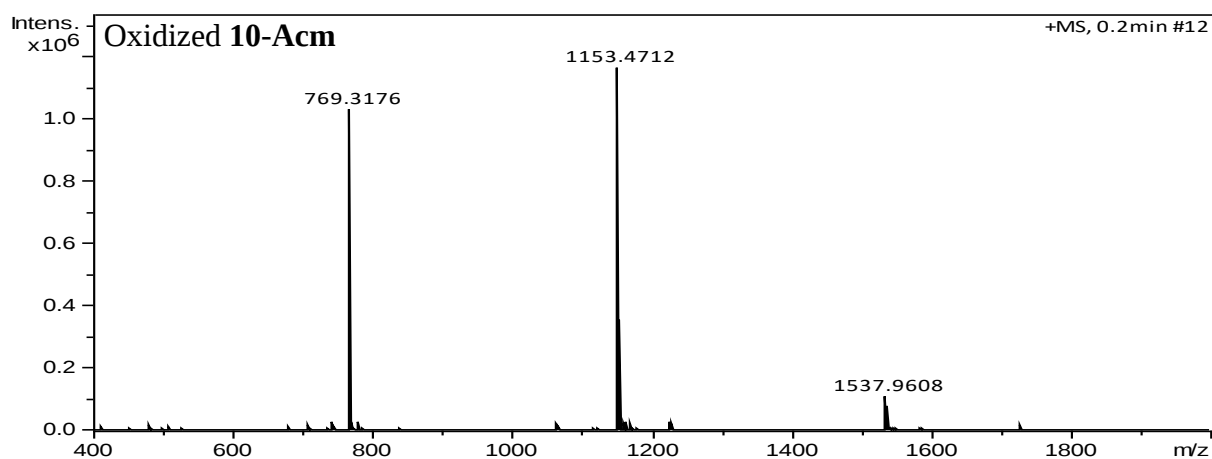


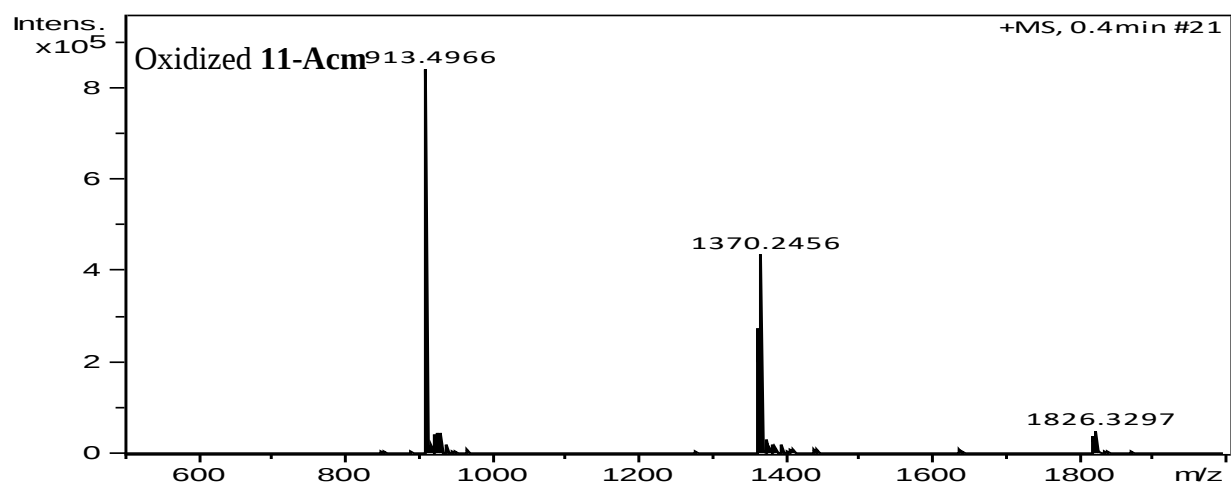
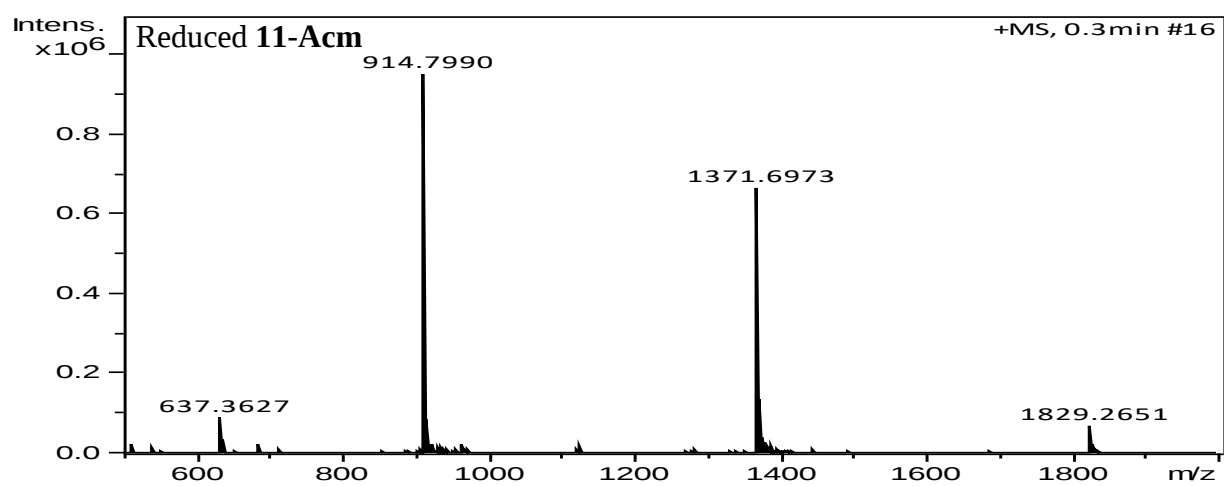
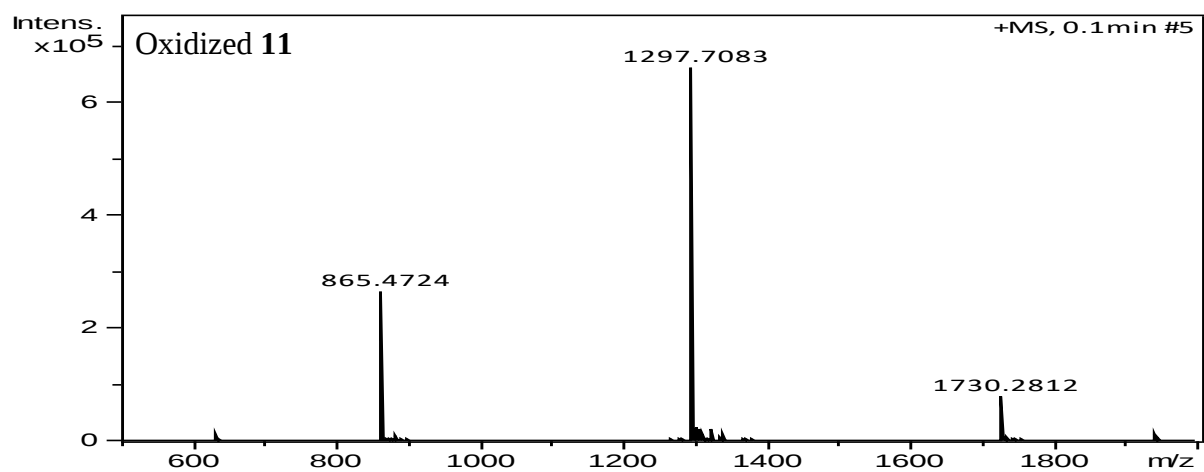


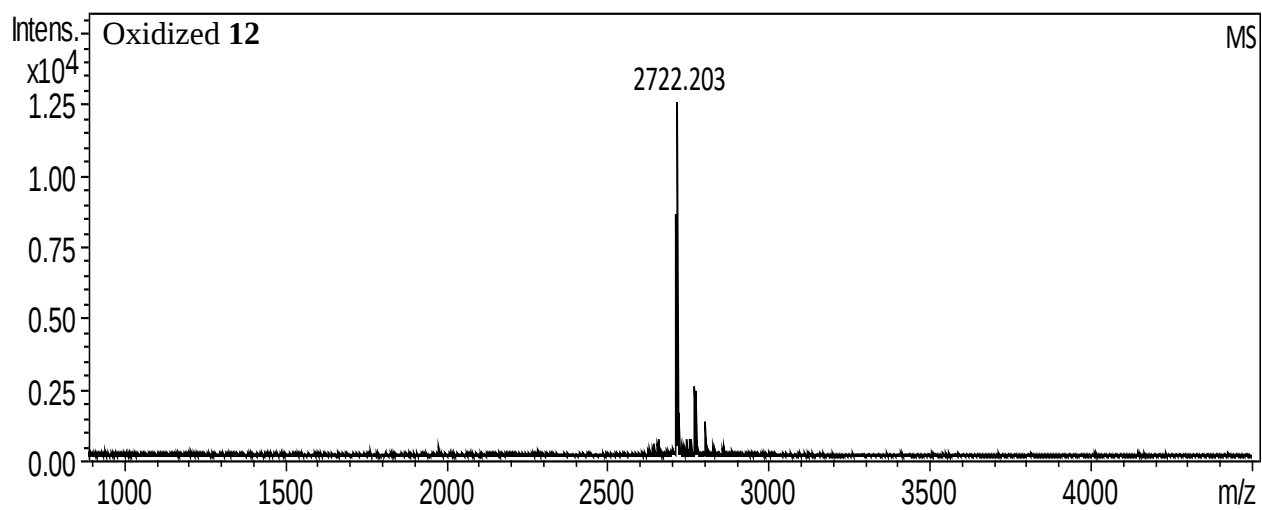
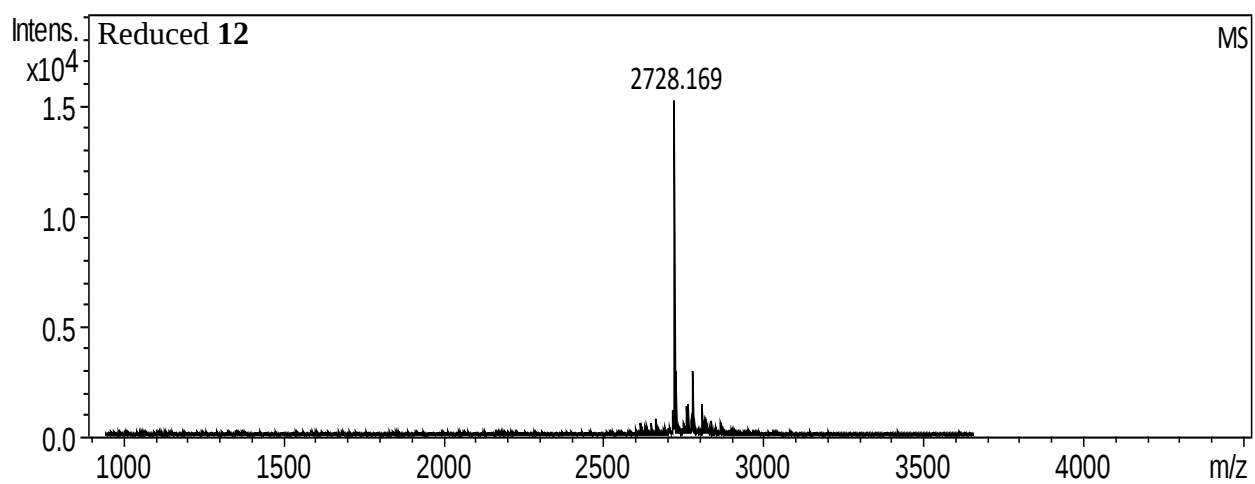
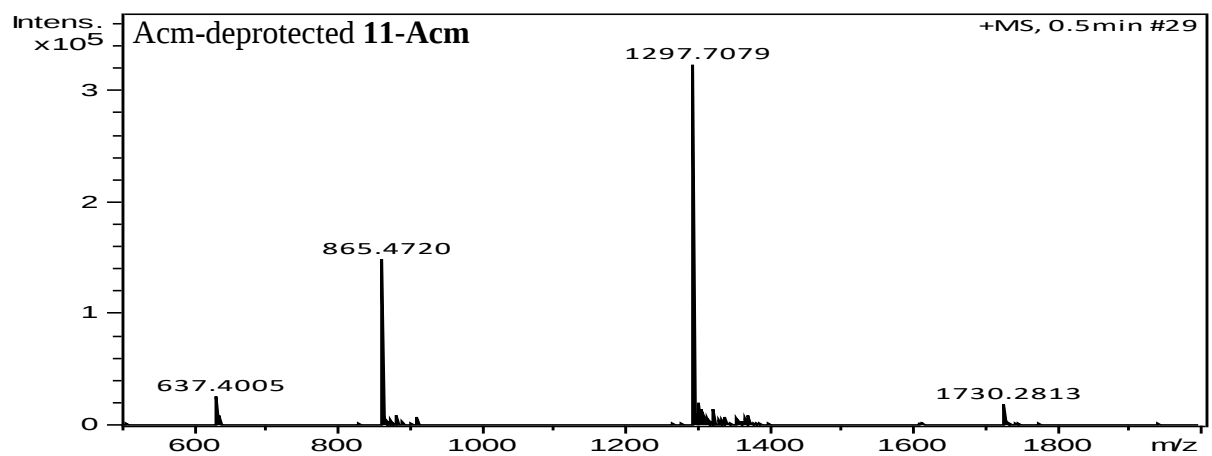


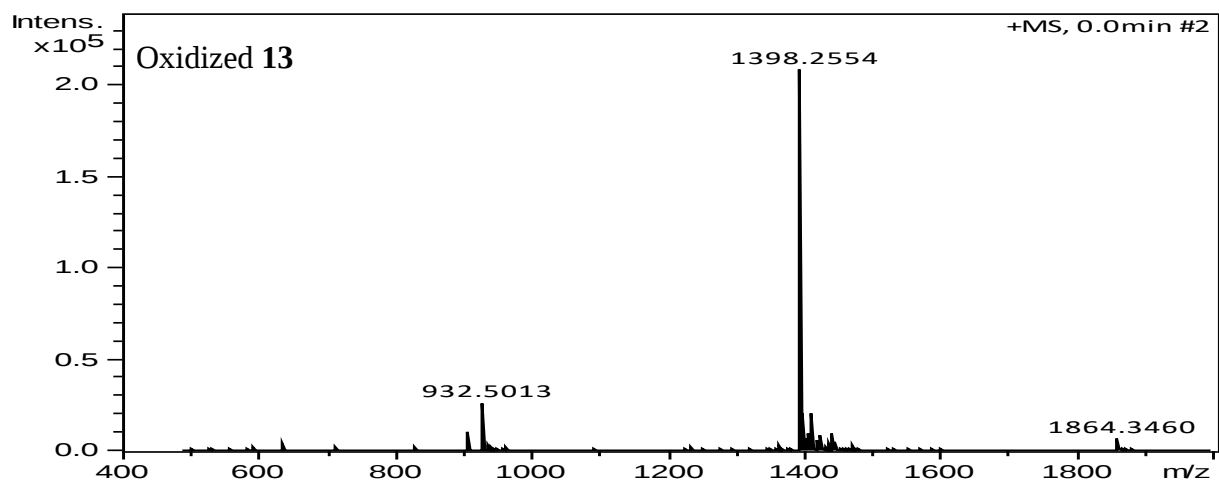
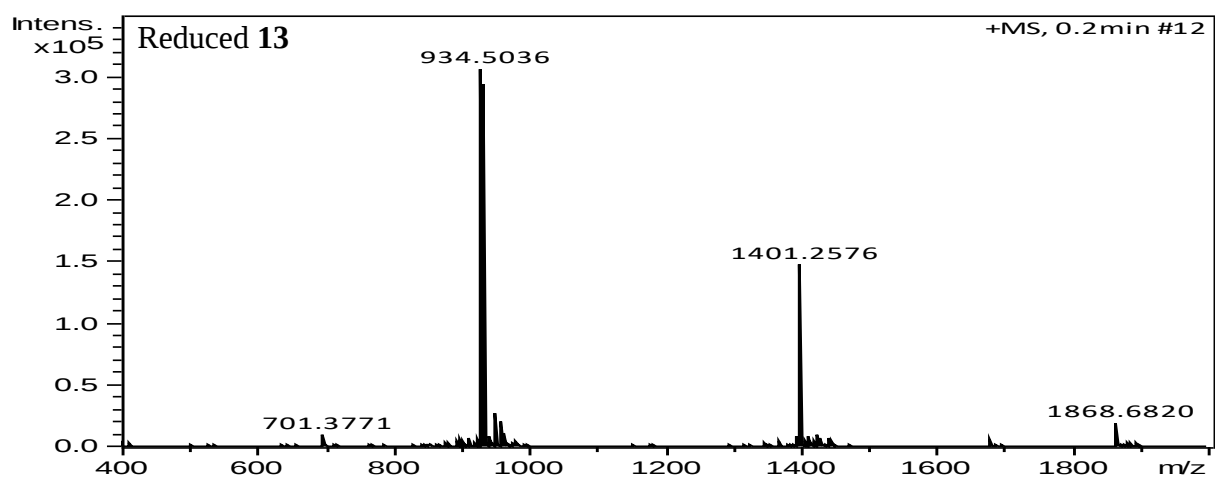
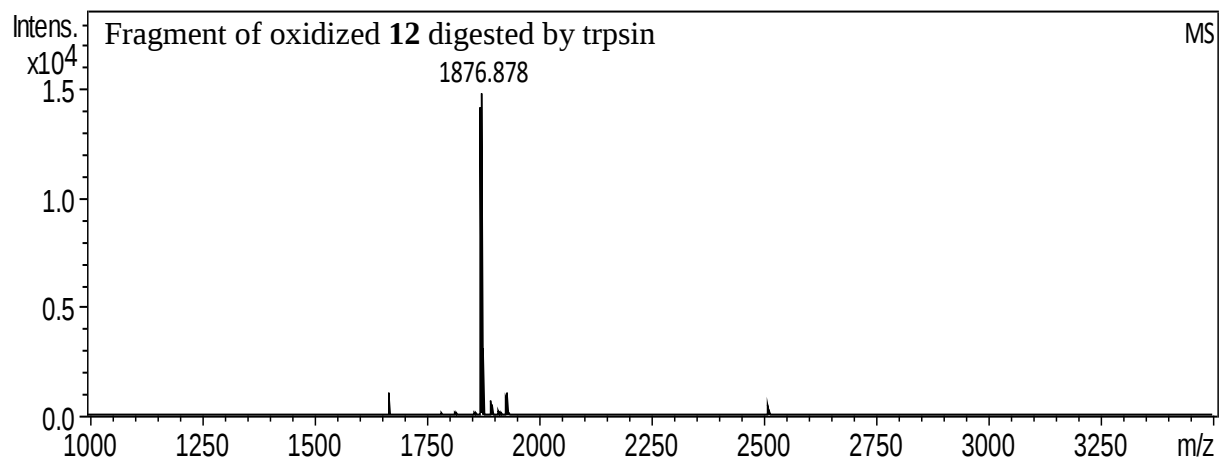


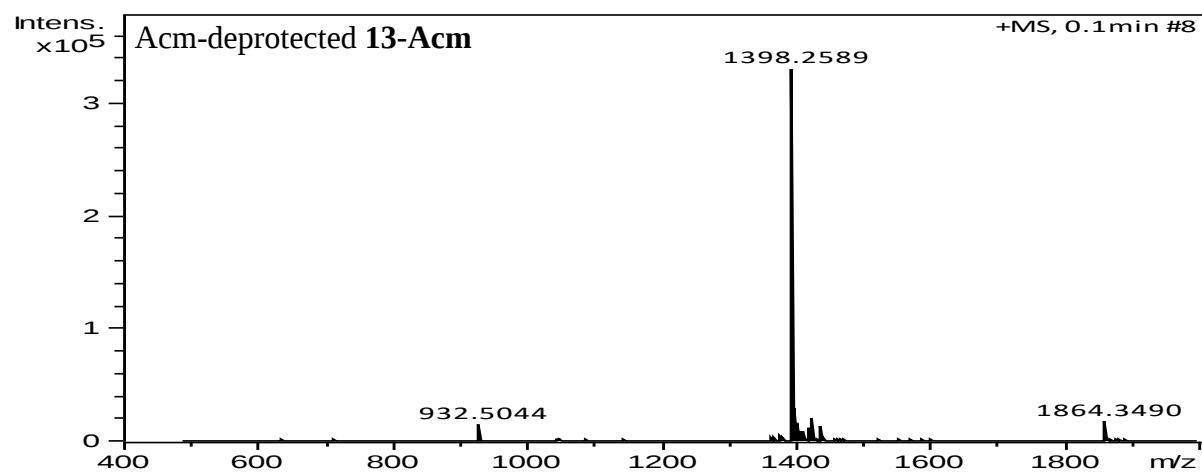
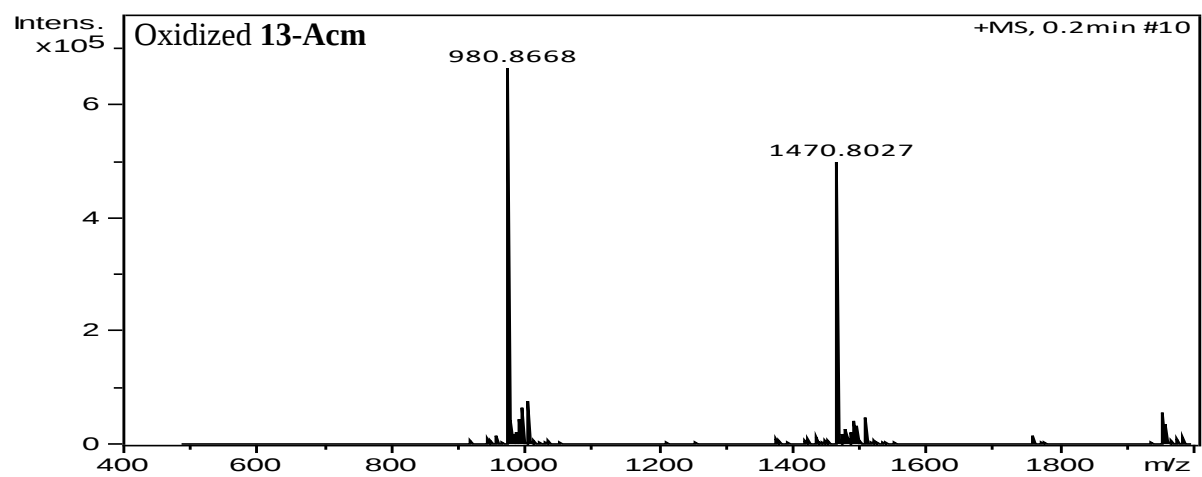
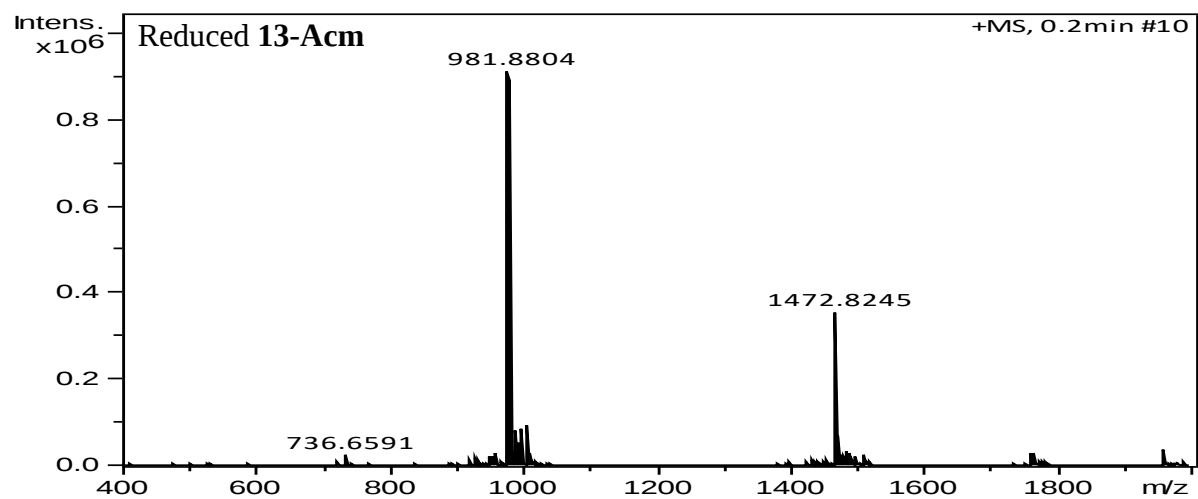


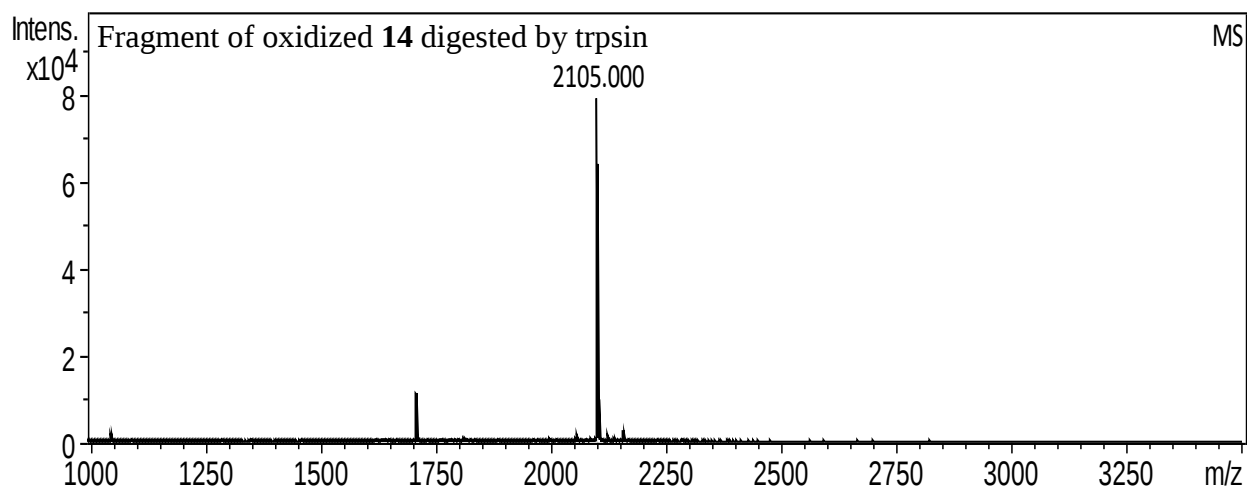
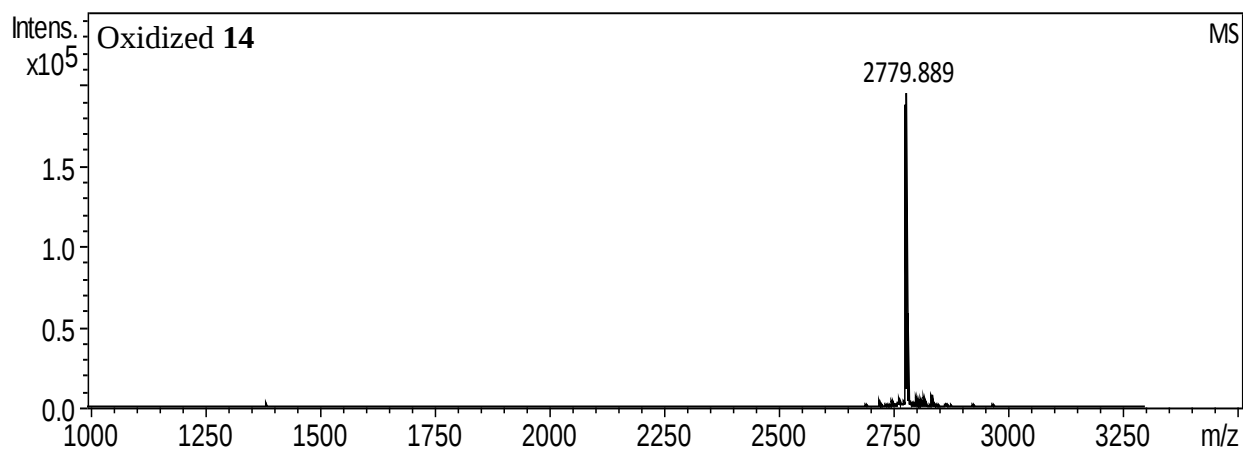
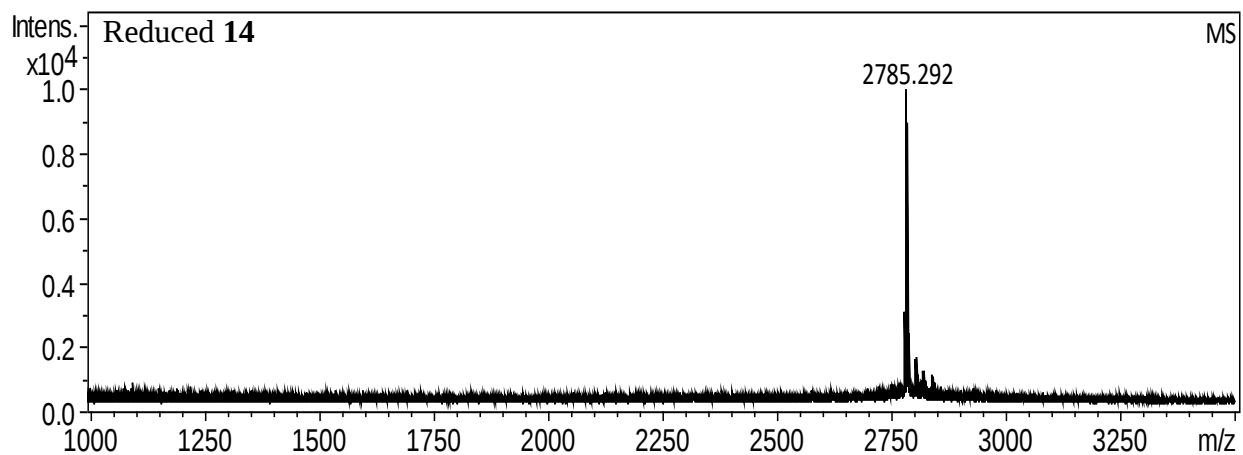


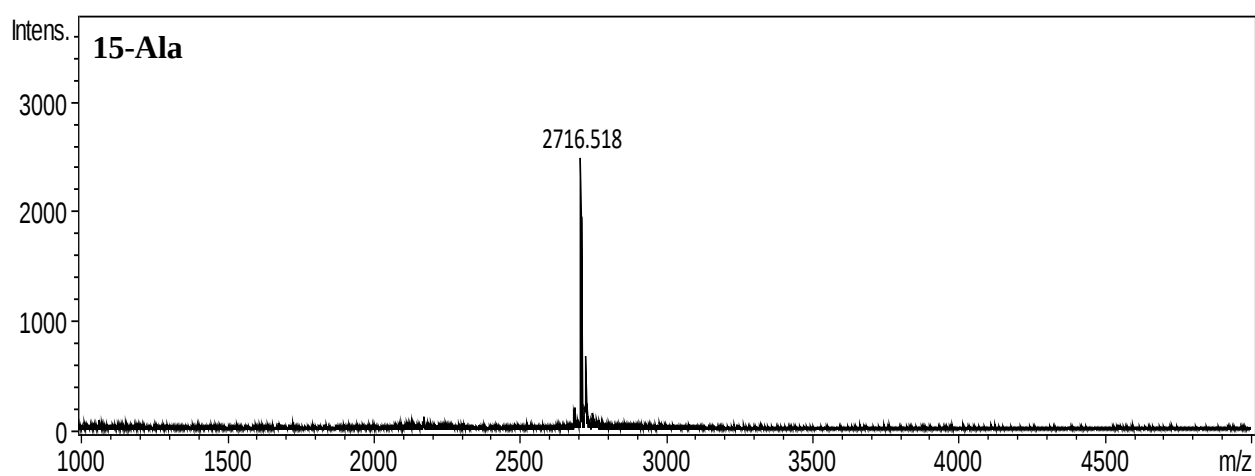
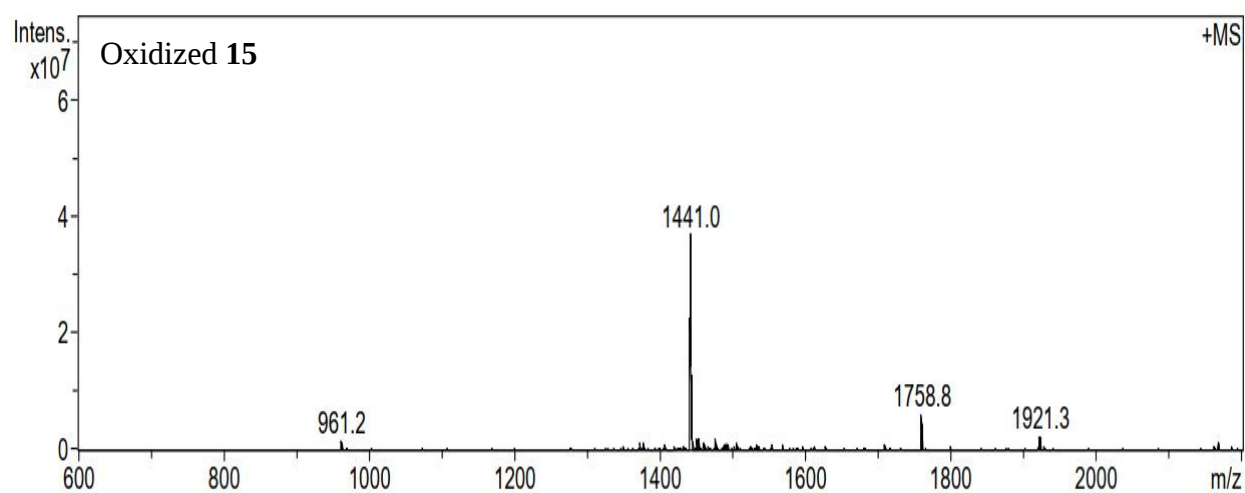
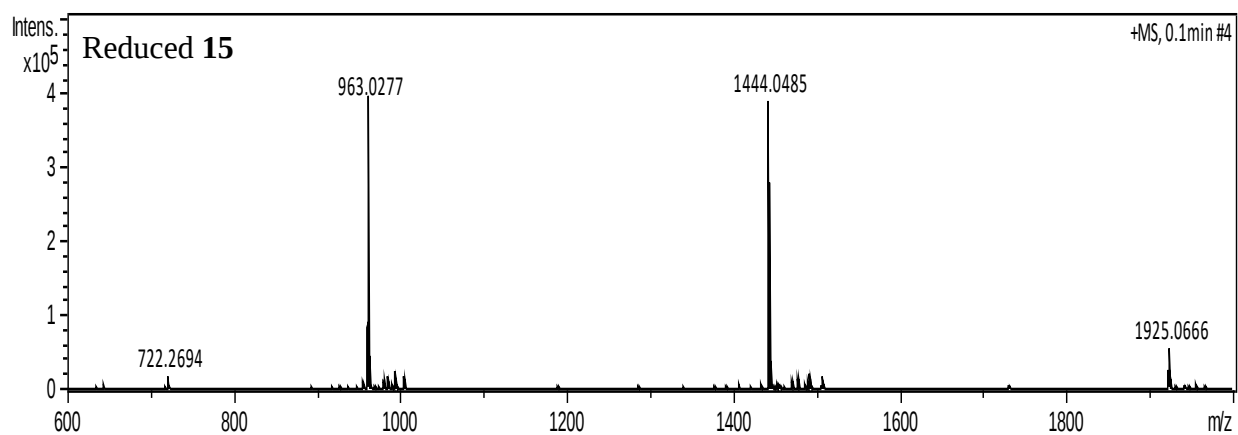


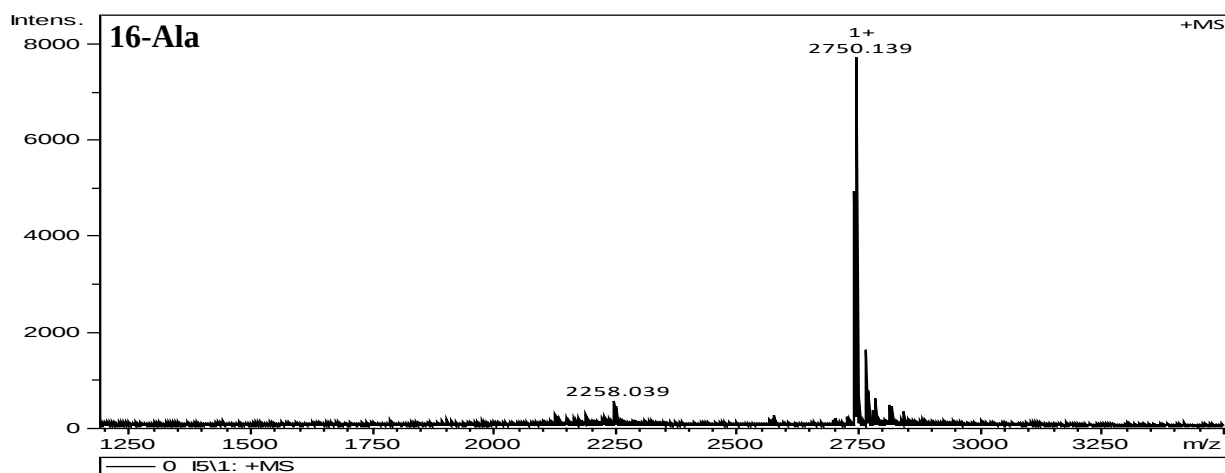
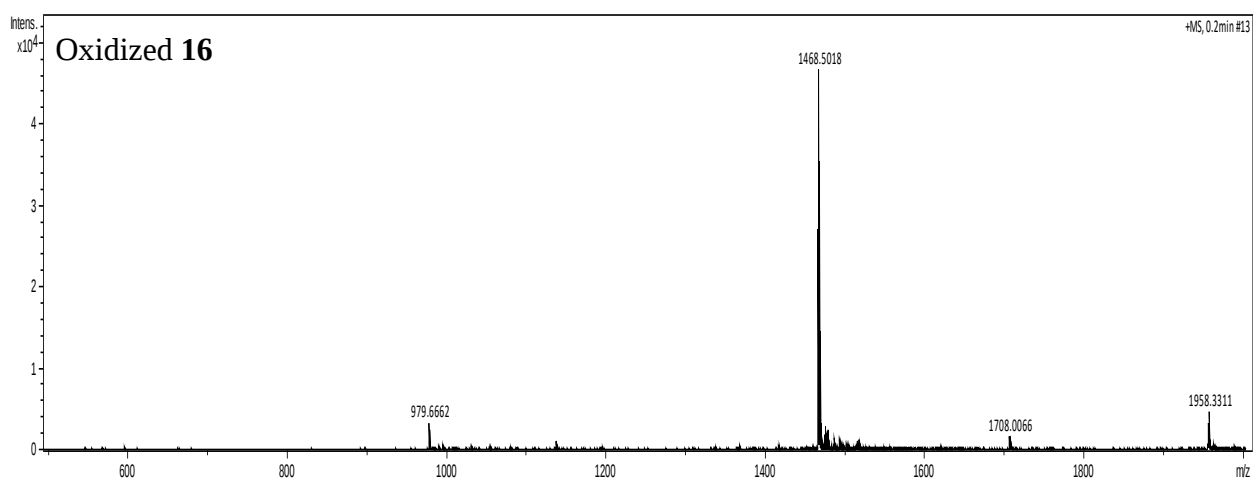
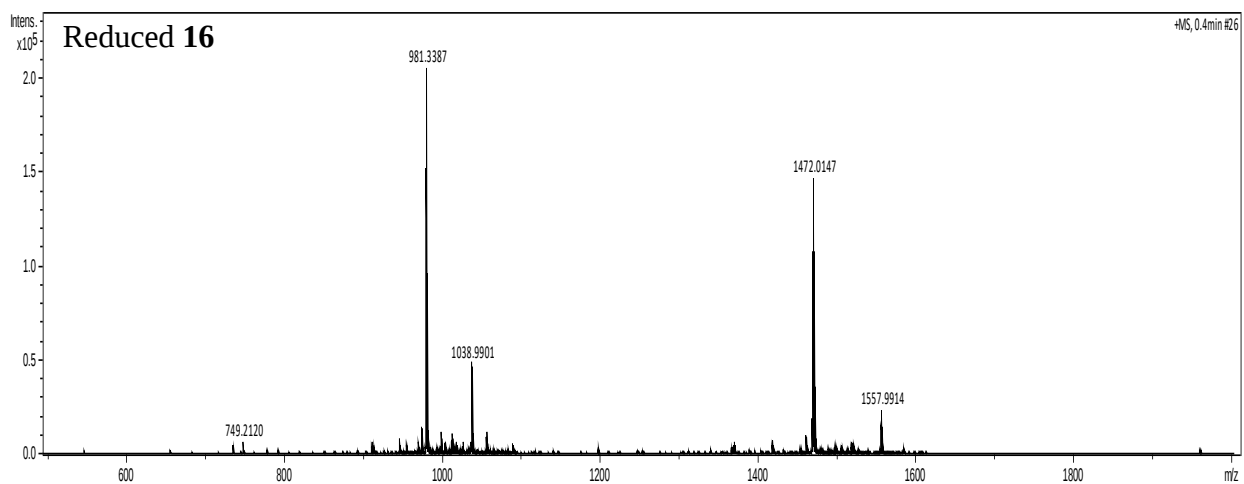


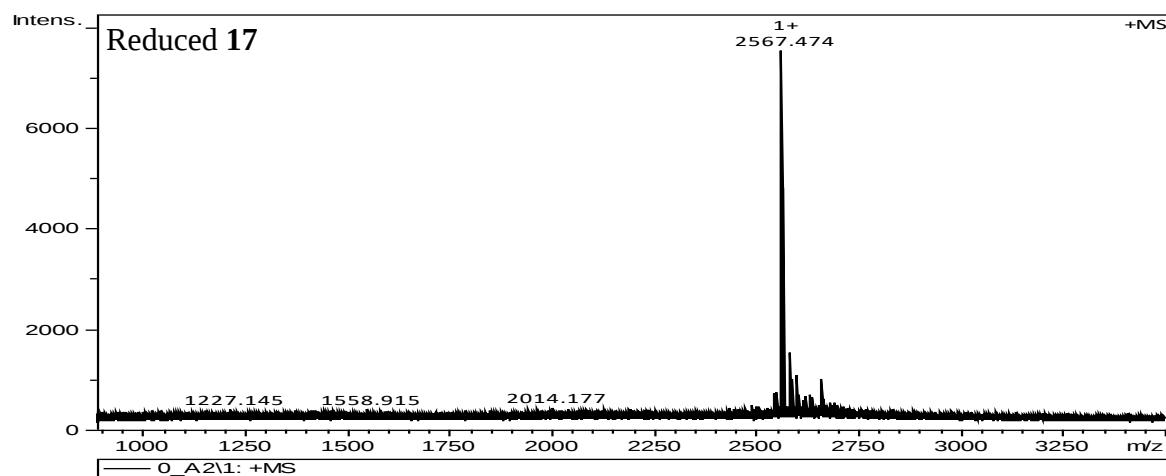
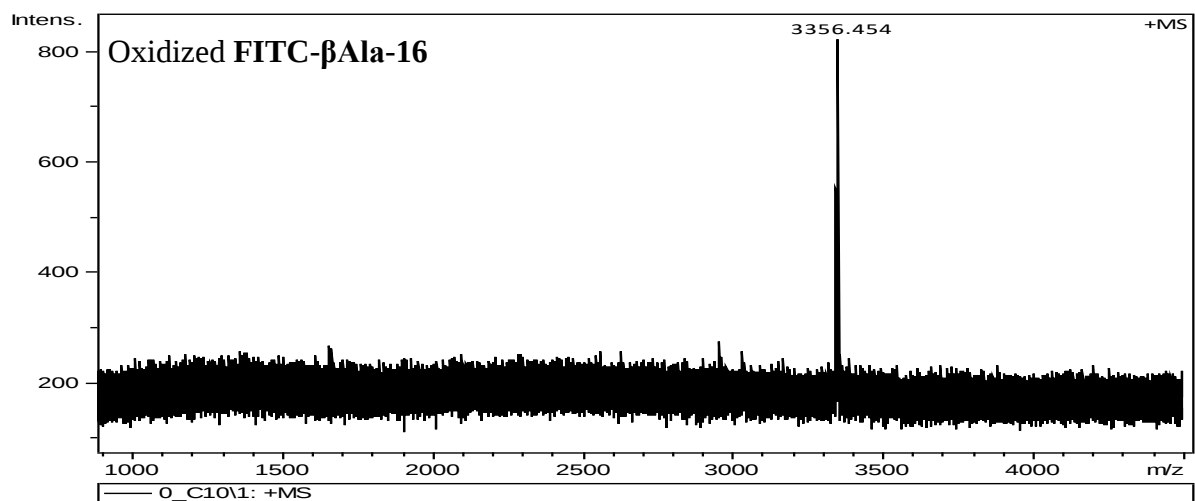
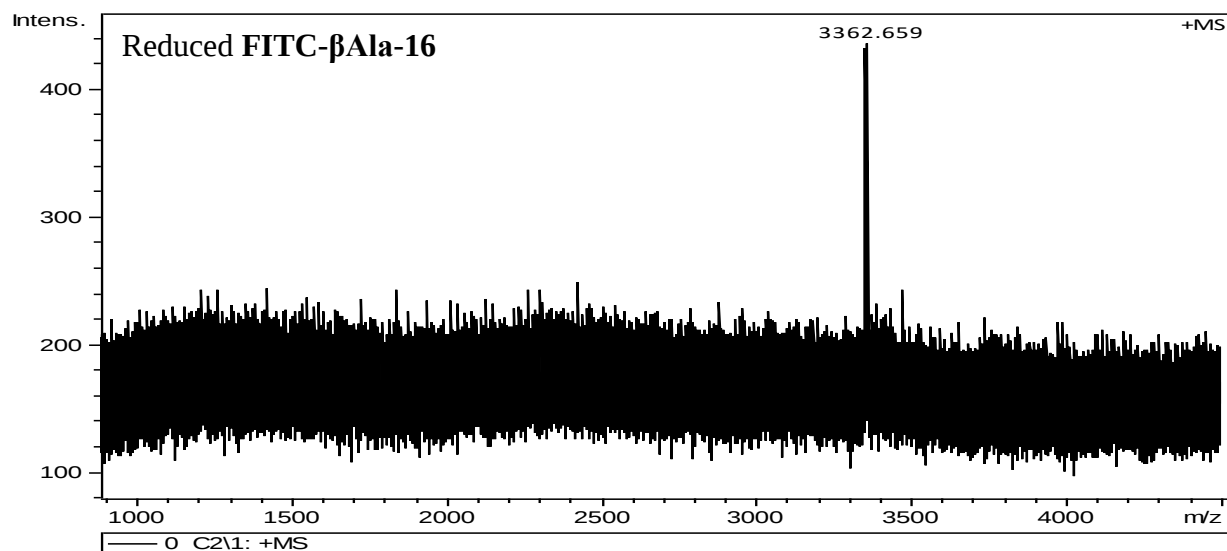


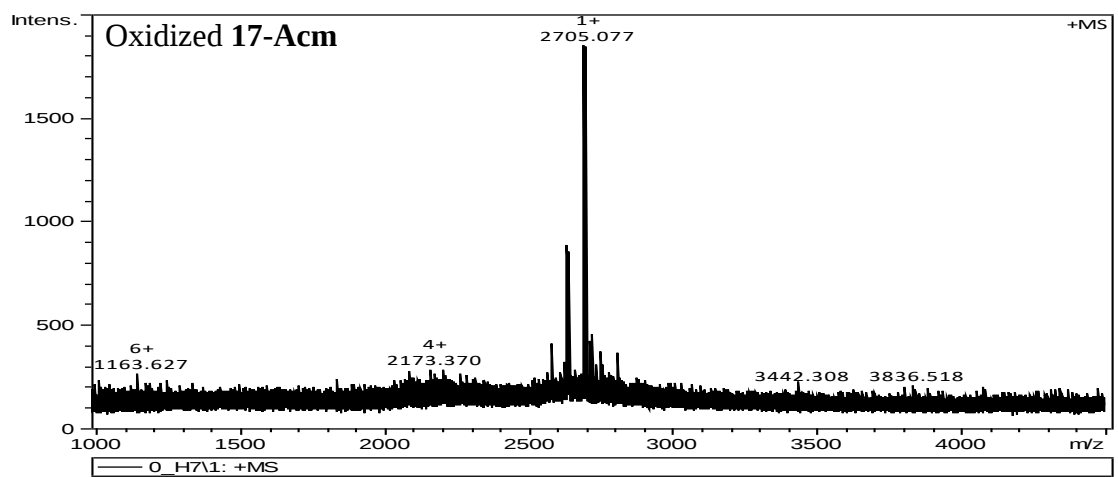
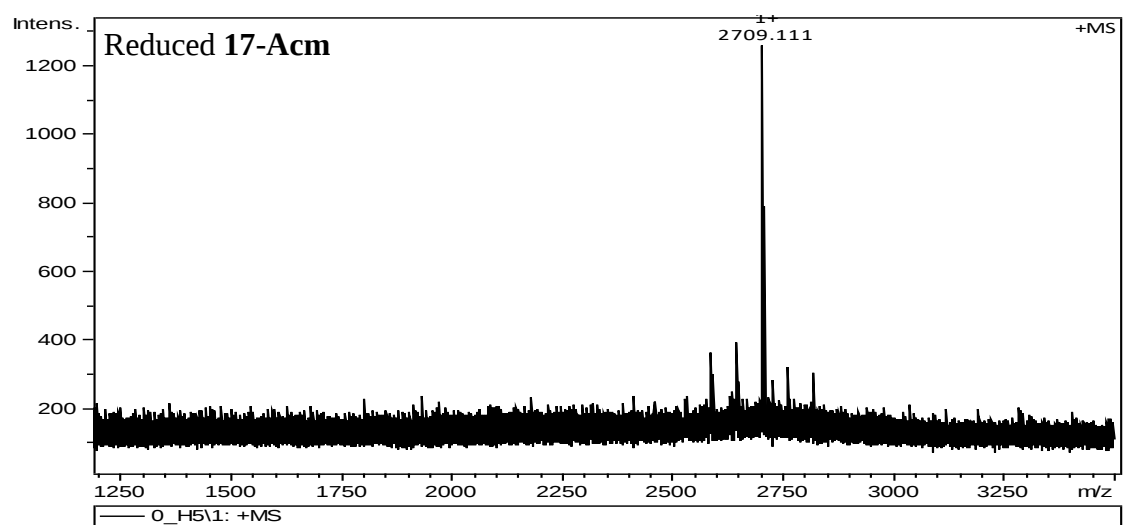
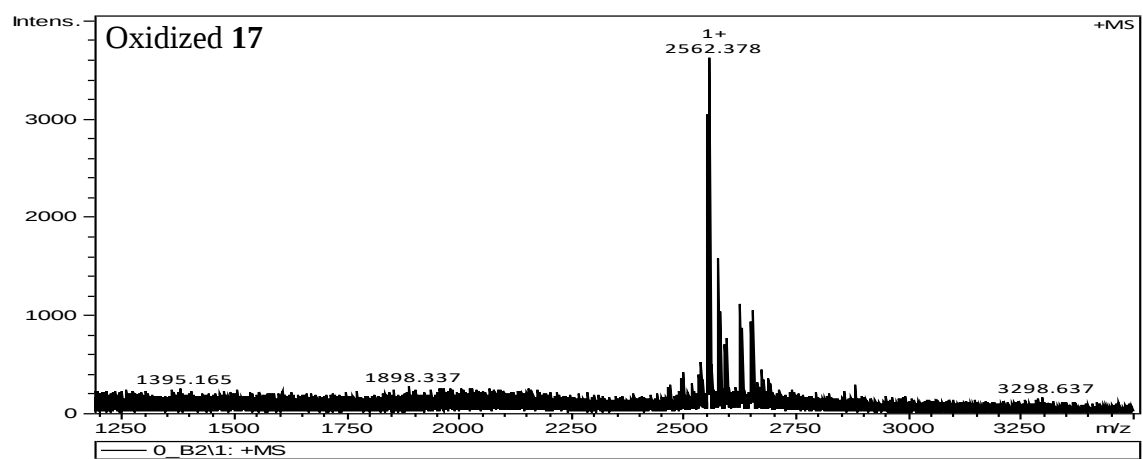












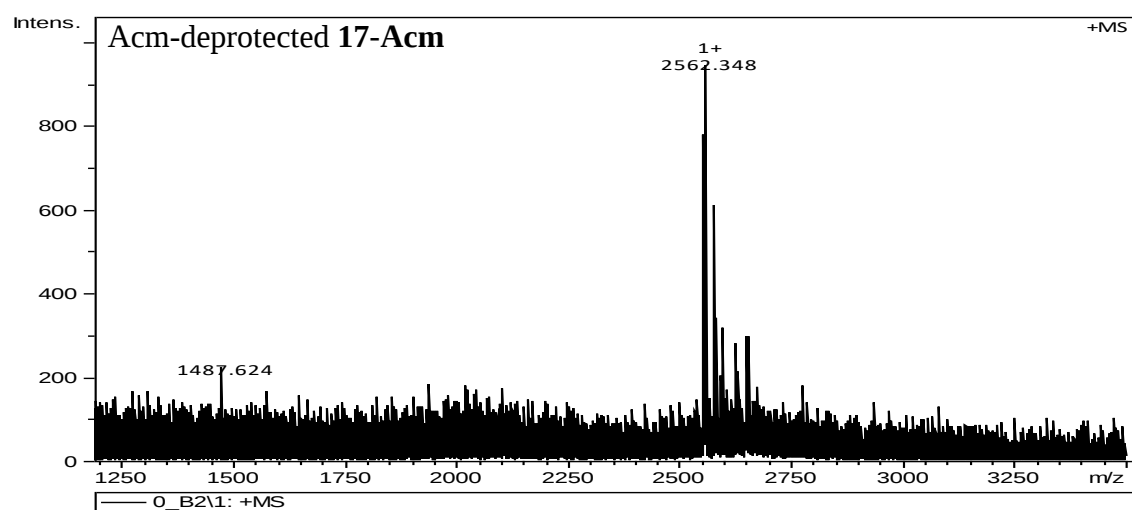


Figure S27. Mass spectra of peptides.

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